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Final Report

R-88-07-0293

Novel Improvements in Production of Alcohols from Surplus Grains

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Project Report # R-88-07-0293

Novel Improvements in Production of Alcohols from Surplus Grains

Principal Investigator: Dr. W.M. Ingledew, Professor *with*
Dr. K.C. Thomas, Professional Research Associate
Dept. of Applied Microbiology and Food Science
University of Saskatchewan
Saskatoon, Saskatchewan
S7N OWO, Canada

Final Report (report #3) , August 18, 1990

a) 1. Executive Summary

Laboratory research was successful in demonstating that distillery mashes made from 100% wheat can be made at sugar concentrations over 35% w/v, and fermented with active dry distiller's yeast or brewer' s yeast slurry to produce over 20% v/v alcohol. Fermentation time can be reduced by the addition of yeast foods containing usable nitrogenous compounds. This allows additional yeast growth which catalyses the conversion of the high amounts of sugar to alcohol at an increased rate. Conditions which require testing in industrial plant scale have been identified, and trials will commence as soon as local industry here has stabilized.

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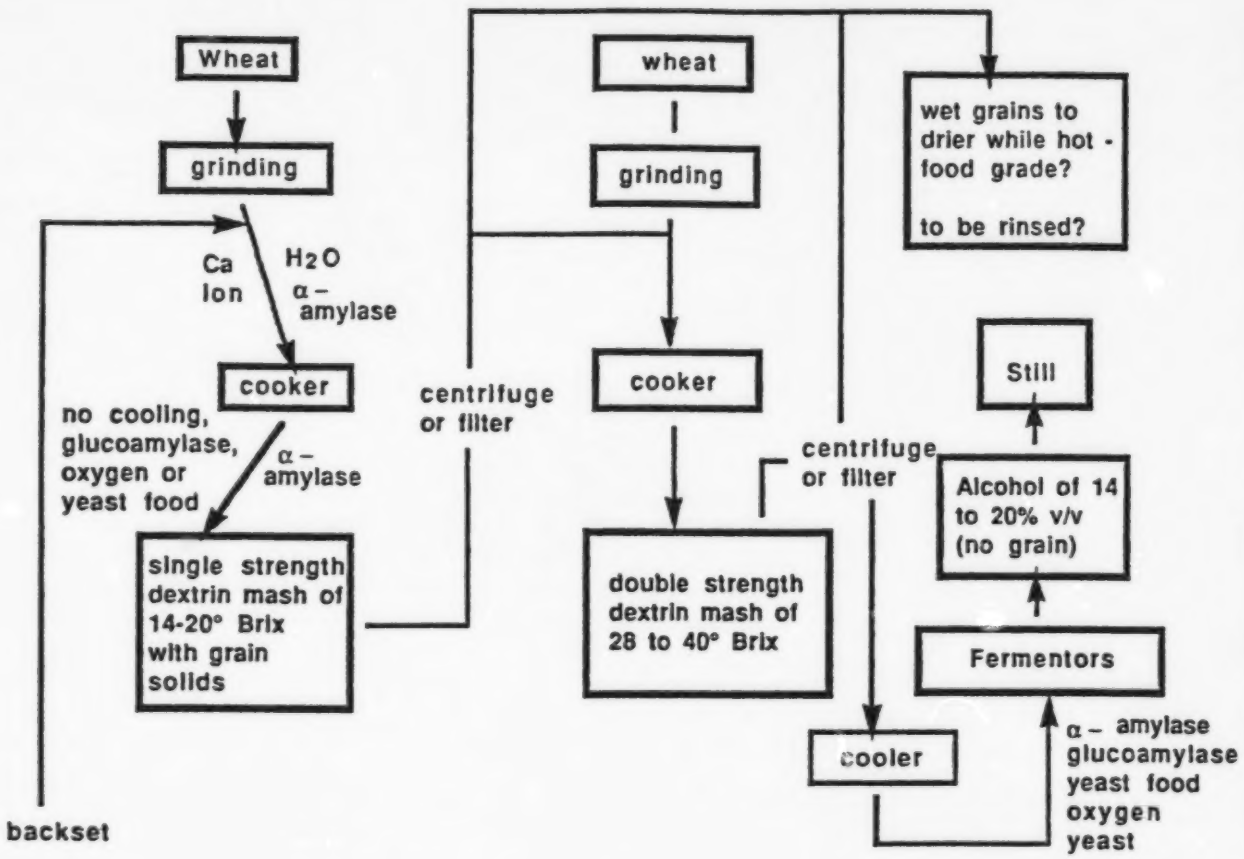
a) 2. Summary of the project

The research carried out under the two years of ADF Grant #R88-07-0293 was designed to be as practical as possible - bearing in mind that one or more alcohol production plants in this province and others outside the province might be able to profit from this work. Furthermore, the source of active dry yeasts and enzymes used by NorthWest Food and Fuel Corp. (Kerrobert) were adopted for the work, wheat was adopted as the sole starch source, and laboratory mashing schemes were kept as close as possible to industrial practice. Having fixed these variables, previous ideas from brewing and winery research in this lab were adapted in order to produce much increased alcohol concentrations in lab fermentations - thus improving plant output using the same capital equipment, labour and nearly the same services.

The two major objectives were 1) to increase ethanol concentration in batch fermentors from the usual 6 to 8% v/v to as high as 20% v/v, and 2) to increase the rate of alcohol production under the conditions required for high alcohol. In addition, 3) this production would be oriented towards a wheat substrate (not corn as in the United States), and oriented to small scale batch production plants.

In summary this laboratory was able to demonstrate:
1) that very high gravity (very high sugar concentration) fermentations (VHG fermentation) of wheat mash was possible and yields of >20% v/v ethanol could be achieved in the lab with normal active dry yeast or brewing yeasts under batch conditions. Modifications of procedures in a fuel alcohol plant would be required to achieve sugar concentrations above currrent industrial levels, to remove the grain prior to fermentation, to add yeast food and oxygen to enhance yeast growth, and to provide adequate cooling. Various measures to accomplish this were suggested involving a "double mashing" and removal of grain at the "front end" enabling its processing for food or other novel end products prior to its degradation and contamination with microbes. This finding dovetails nicely with work in progress in the labs of Drs. F. and K. Sosulski. A double concentration of carbohydrate can then be obtained. A flow scheme has been developed and is shown here. It has yet to be tested under industrial conditions. Without such modifications, higher alcohol via VHG fermentation cannot be obtained.



Scheme for Industrial Production of Alcohol by VHG Technology

w.m. Ingledew, 1990

2) that VHG fermentations must be conducted at temperatures of 20-25° C or below. Alcohol produced at higher temperatures is toxic to yeast cells at a lower concentration. This limits the exploitation of VHG technology to temperate climates or to those plants in tropical areas where cold water or refridgeration is available at reasonable cost. Even at these temperatures, VHG fermentations were complete in < 72 hours. Plant scale trials are needed to assess applicability and cost of these procedures in industrial practice.

3) that VHG fermentation times are reduced by the addition of yeast foods. Work concentrating on the amount and type of nitrogen sources which stimulate wheat starch fermentation was done using inexpensive (crude) sources like yeast extract as well as purified sources of amino acids - all in an attempt to understand the yeast's requirements. Some amino acids actually inhibit fermentation - this phenomenon was investigated in more detail for publication in the scientific literature. To lower alcohol production costs, neutrase (a proteolytic enzyme) was shown to be effective in stimulation of VHG fermentation - lowering the requirement for yeast extract additions. This enzyme liberated amino acids from soluble wheat proteins in the mash. Mineral were not required as supplements, having no stimulatory effect in fermentation.

4) that VHG wheat mash fermentations could be carried out using active dried yeast (conveniently supplied at low cost by yeast companies - avoiding the need for subsequent microbiological expertise in the plant) or by liquid yeast slurries supplied by brewers. No specially engineered yeasts or isolates were required.

5) that if no or low amounts of yeast foods were used, higher pitching (inoculation) rates of yeast (or recycling of yeast) resulted in faster fermentation; fermentations completed earlier. With yeast food addition, such differences were less defined.

6) that addition of yeast foods had no effect at 30 and 35 ° C, and did not protect yeasts from the effects of ethanol at these temperatures. Yeast death still occurred.

7) that a thermotolerant yeast claimed by Labatts Brewing Co. to be useful to ferment at temperatures near 40° C was shown to be of no use in VHG fermentation.

The results of this work (detailed summary of the last year of research follows) are suitable for publication. One manuscript has already been published (section c), a second is prepared and will be submitted soon, and others may be forthcoming. In addition, WMI was asked to write a major chapter in Volume 5 of a series "Industrial Microbiology" edited by Dr. A.H. Rose. It is entitled "Yeasts for Production of Fuel Ethanol". These publications along with those from almost 18 years of research in alcohol related matters have been of great interest to the alcohol industry in Canada and the US, and the results of VHG technology are under test and exploited in the wine, beer and hopefully the fuel alcohol industries.

The ADF in thanked for its support of research in this area in the Department of Applied Microbiology and Food Science at the University of Saskatchewan. We hope soon to be able to apply some of these ideas in full plant scale production through an IRAP grant recently awarded to NorthWest Food and Fuel Corp. , or in a molasses /grain mixed substrate with GPCP Technologies Inc.

b) Technical Report

Background and Objectives

The objective of this research was to apply the Very High Gravity fermentation technology developed in this laboratory to fuel alcohol production and in the process, develop techniques usable in industry to convert Saskatchewan grown grains to industrial alcohol in the most efficient and economical way. Most of our objectives have been realized and the following sections highlight our major findings.

In preliminary studies, three sources of wheat were compared with regard to their suitability for fuel alcohol production. There were no noticeable differences among these sources in their fermentability or final yield of ethanol. For this reason only one, Schmidt wheat, was used in the rest of this study, and the conclusions drawn from these studies can be applied to others.

Experimental Methods

Various experimental procedures used in this study have been described in the reports submitted earlier (Report #1, December 8, 1988; Report #2, July 10, 1989). For the sake of easy understanding of the results, a brief overview of the experimental procedures are given below.

Grinding and Mashing of wheat. Wheat was ground with a plate grinder (Falling Number AB, Type KT-30, Stockholm, Sweden) at setting number 2. For mashing, 700 g of ground wheat was dispersed with constant agitation in 2100 ml of hot (60°C) water containing 10 μM calcium chloride. To this, 3.5 ml of the HT α-amylase preparation was added. After 5 min, the temperature was raised to 95-97° C and held for 60 min with continuous stirring. The volume lost due to evaporation was made up by adding sterile distilled water and the temperature was then lowered to 80° C. The gelatinized starch was liquefied further by the addition of another 3.5 ml of high-temperature α- amylase and incubated at 80° C for 30 min. This liquefaction step hydrolyzed all the starch into soluble sugars and dextrins. The mash thus prepared contained 19 to 20 g of dissolved solids per 100 ml (water soluble portion). This mash was saccharified and fermented directly or its dissolved solids content was further raised by adding freeze dried "wheat hydrolysate" (preparation outlined below) and then used for fermentation.

In most experiments, 500 g aliquots of the wheat mash were transferred to jacketed sterile Wheaton Celstir fermentors. If additives were used, they were sterilized in these fermentors by autoclaving at 121° C for 15 min. The fermentors

containing the mashes were connected to a Haake D3-G water bath circulator (Haake Inc. Saddle Brook, NJ) maintained at 30° C. To each fermentor, 1 ml of Allcoholase II (glucoamylase) was added to saccharify the dextrins to fermentable sugars. After 30 min the temperature of the fermentors was lowered and maintained at 20°C throughout the fermentation.

Preparation of inocula: Eleven grams of active dry yeast were dispersed in 99 ml of prewarmed (38° C) 0.1 % sterile peptone water. After 20-25 min at 38° C the required volume of inoculum was added to each fermentor.

Preparation of "wheat hydrolysate": Liquefied (but not saccharified) wheat mash was strained through a double layer of cheese cloth and the clear liquid was centrifuged (10,300 x g for 15 min) to remove the remaining suspended particles. The resulting supernatant liquid was freeze dried. The freeze dried product, called "wheat hydrolysate" was used to raise the dissolved solids contents of wheat mashes when very high gravity (VHG; in this case 25 g or more dissolved solids per 100 ml) fermentations were attempted.

Determination of total solids. Total dissolved solids were determined by estimating the specific gravity of the water soluble portion of the mash obtained by centrifugation at 10,300 x g for 15 min. The specific gravity was determined at 20° C with a digital density meter (DMA-45, Anton Paar, Graz, Austria). With the aid of appropriate tables the results were converted to grams of dissolved solids expressed as sucrose per 100 ml.

Free amino nitrogen (FAN): The FAN in supernatant liquid was determined by the ninhydrin method of the European Brewing Convention. Amino acids (free as well as hydrolyzed) were quantitated by a Beckman amino acid analyzer or by a Waters high performance liquid chromatograph (HPLC).

Ethanol. Ethanol was determined by the alcohol dehydrogenase assay (Sigma Bulletin No. 331 U.V. Sigma Chemical Co. St. Louis. MO.). This method overestimates ethanol by about 7 % as shown by assays of standard solutions of ethanol (unpublished results) . All values reported here have been corrected for this overestimation.

Cell number and percentage of viability. Total cell counts and viable cell counts were determined by the direct microscopic method at a magnification of 400 X with an improved Neubauer counting chamber. Samples were diluted with modified Ringer's solution containing 250 mg methylene blue per liter. Modified Ringer's solution contained per liter: 8.6 g NaCl ; 0.3 g KCl ; 0.33 g $CaCl_2$; 0.5 g $Na_2S_2O_3 \cdot 5H_2O$ and 10.0 g sucrose. Cells which stained blue were considered to be non viable. Results

are expressed as cells per g mash rather than per ml because it was not possible to pipette the viscous mash accurately.

Results and Discussion

I. Relationship between the quantity of assimilable nitrogen and fermentability of wheat mash

An all-malt brewery wort of 16° P (where ° P corresponds to grams of dissolved solids expressed as sucrose per 100 g of wort) would normally contain about 250-300 mg FAN per liter. Mashes prepared from wheats which contained similar starch contents had only 54-58 mg FAN per liter. In brewing, adequate rates of attenuation and complete utilization of fermentable sugars require 150 mg FAN per liter in a 12° P wort (or 200 mg FAN per liter in a 16° P wort). If the FAN content is less than this value, the wort is likely to be deficient in nitrogen and incomplete or protracted (stuck or sluggish) fermentations could result. Using such criteria, wheat mash might be considered severely limited in utilizable nitrogen.

Wheat mashes (16 g dissolved solids /100 ml) were inoculated with active dry yeast to give an initial cell number of 1.75×10^7 per g of mash and fermented at 20° C with stirring. Virtually all of the fermentable sugars were exhausted by 120 h (Fig.1), - a result unattainable in "wort" of similar gravity with such low levels of nitrogen. Nearly 80% of the total 58 mg FAN per liter was taken up by the yeast. Most of this uptake of nitrogen occurred during the early stages of fermentation. The rate of fermentation of wheat mash could be stimulated either by providing the yeast with extra amounts of utilizable nitrogen through *in situ* hydrolysis of wheat protein, or by adding an external source of FAN such as yeast extract. Wheat protein was partially hydrolyzed by adding 0.02 %(v/v) of a commercial protease ('Neutrase') to the fermentor 30 min after inoculation. Addition of the protease reduced the fermentation time from 120 h to less than 72 h (Fig. 1).

The stimulatory effect of Neutrase apparently resulted from the increase in available FAN (used for yeast growth) and not through addition of other growth factors such as vitamins and minerals in the enzyme preparation (verified with a boiled enzyme control). The observed stimulation of fermentation was apparently mediated through increased growth and cell multiplication.

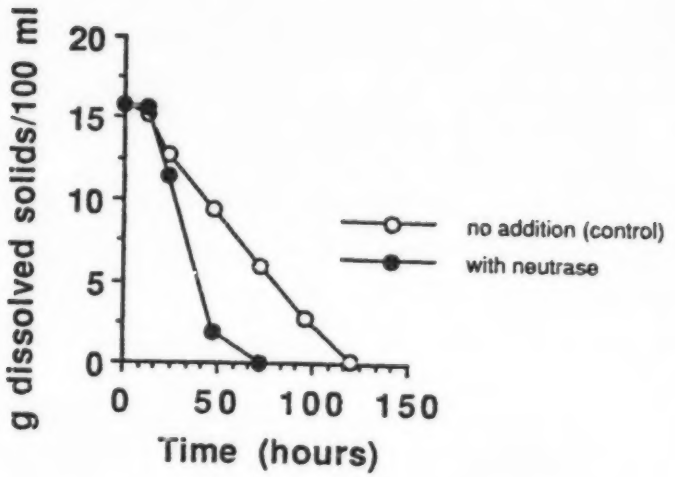


FIG. 1. Changes in dissolved solids (mostly sugars) during fermentation by active dry yeast at 20°C

Without protein hydrolysis, a maximal yeast cell number of 1.5×10^8 per gram of mash was reached within the first 24 h and there was no appreciable change thereafter (Fig. 2). When extra free amino nitrogen was made available through protein hydrolysis, the cell population reached 4.1×10^8 per g of mash by 48 h.

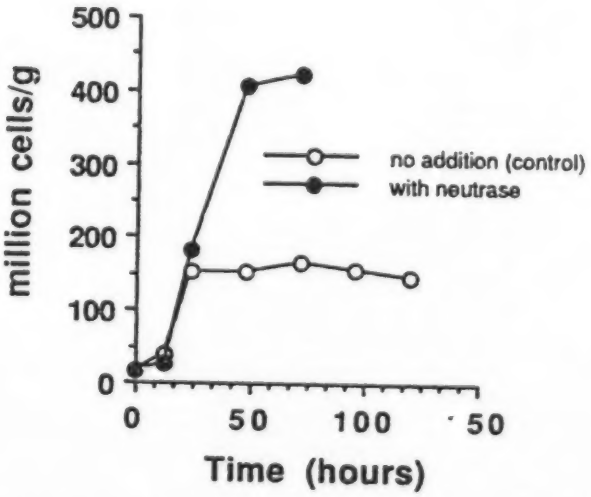


FIG. 2. Changes in the number of yeast cells during the fermentation of wheat mashes by active dry yeast at 20°C.

The unsupplemented wheat mash contained insufficient amounts of FAN to promote cell growth to the extent required for a fast rate of attenuation and for complete conversion of fermentable sugars to ethanol. Addition of utilizable nitrogen in the form of yeast extract, casamino acids or even a single amino acid such as glutamic acid was also found to stimulate the rate of attenuation (Fig. 3). For this study, very high gravity mashes (25.7 g dissolved solids 100/ml) which contained 74.4 mg of FAN per liter were used. Supplementations were made at the rate of 3.0 g of the selected nitrogen source per liter of mash. Without supplementation, the attenuation rate was slow and a small amount of sugar remained even after 144 h. Addition of yeast extract or glutamic acid resulted in complete utilization of the sugar within 96 h. Casamino acids were not as stimulatory as yeast extract or glutamic acid, although fermentations with this additive were still considerably faster than that observed with the control. A single amino acid such as glutamic acid was as effective in stimulating growth as the mixture of amino acids contained in the yeast extract. It must be pointed out, however,that more free amino nitrogen is contained in a given weight of glutamic acid than in either yeast extract or casamino acids. No attempt was made to balance FAN because not all FAN in casamino acid or yeast extract preparations is usable.

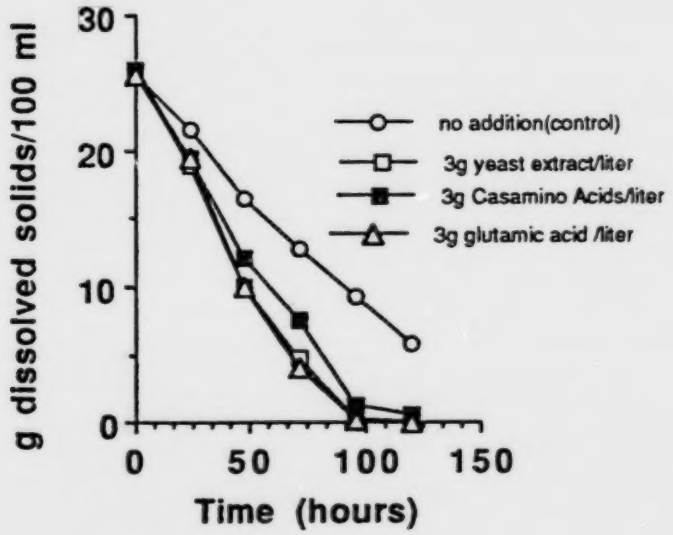


FIG. 3. Changes in dissolved solids contents during the fermentation of wheat mashes supplemented with various nitrogen sources.

The low level of nutrient supplement (135-240 mg FAN per liter) was insufficient to promote fermentation at the fastest rate. Separate experiments showed that when wheat mash was supplemented with increasing amounts of yeast extract the fermentation rate increased. The fermentation was completed within 72 hours even when the wheat mash contained 31 g of dissolved solids if the amount of yeast extract supplement was raised from 0.3 % to 0.9 %. Nearly 80 % of the available FAN was used by the yeast and virtually all of the FAN uptake occurred during the first 24 h, when the initial FAN value was low. The uptake period extended to 48 h when the initial FAN values were higher (Table 1).

Table 1. Effect of various concentrations of yeast extract on the uptake by yeast of free amino nitrogen from wheat mash containing 31 g dissolved solids per 100 ml.

Hours	Amount of yeast extract added to wheat mash (g/liter)			
	0	3.0	6.0	9.0
	mg FAN per liter			
0	113	283	434	593
24	31	63	215	355
48	29	32	37	43
72	25	30	35	48
96	24	31	38	59
144	23	30	ND	ND
Total taken up[1]	90	253	399	549
% taken up in 24 h.	90.9	86.9	54.8	43.3

[1] These amounts are considered as 100 % for the calculation of % used in first 24 h.

In the unsupplemented wheat mash, the cell number increased from 1.5×10^7 to 2.18×10^8 within the first 48 h. There was no significant change in cell number after this, although 55% of sugar was fermented after cell proliferation had ceased. The maximum cell number in each case appeared to be a function of the initial FAN values. With 0.9 % yeast extract, there was continuous growth throughout the fermentation period and a maximum cell number of 4.0×10^8 was reached towards the end of fermentation.

Wheat mash was not deficient in any minerals. Supplementing the wheat mash with phosphate, magnesium or with other trace elements (Zn, Mn, Fe, Co, Mo, Cu, B, and I) did not affect the growth rate or the attenuation rate (data not shown). The nature of the amino acid which served as the source of nitrogen did, however, influence the rate of fermentation and growth. This was made clear by an experiment in which glycine was used as a source of nitrogen. The unsupplemented mash contained 97.7 mg FAN per liter and 35 g of dissolved solids per 100 ml. Without any added nutrient, 90.6% of the dissolved solids was used up by the yeast within 8 days (Fig.4). This indicated that if the sugar concentration was raised above 30% (w/v), the fermentation time would increase significantly.

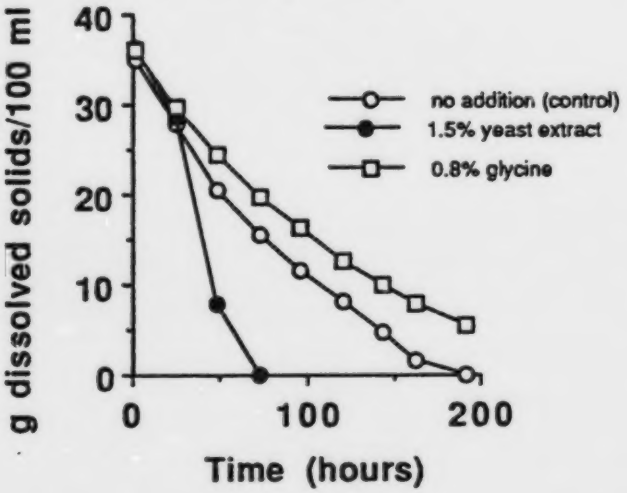


FIG. 4. Changes in dissolved contents during fermentation of wheat mashes supplemented with yeast extract or glycine.

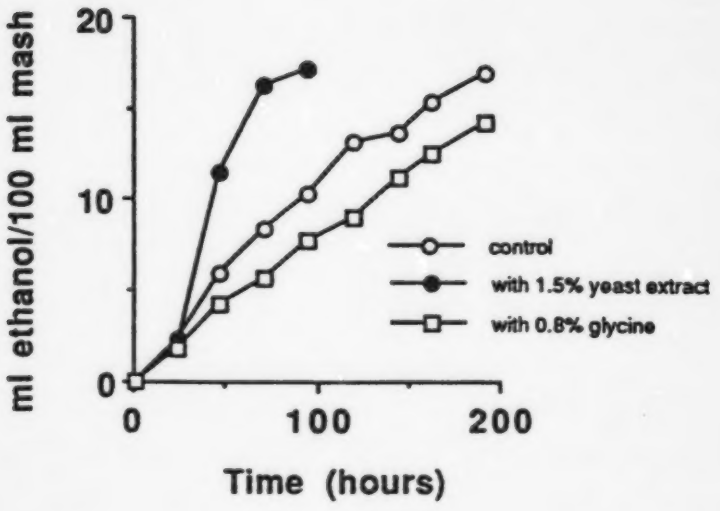


FIG. 5. Production of ethanol from very high gravity wheat mashes supplemented with yeast extract or glycine.

Ethanol production paralleled the attenuation rate (Fig. 5). An ethanol yield of 17.1 % (v/v) was obtained within 3 days when the wheat mash was supplemented with yeast extract. In the absence of nutrient supplement, the fermentation rate was slow but a final ethanol yield of 16.9% (v/v) was still achieved after 8 days. Supplementing the wheat mash with 1.5% (w/w) yeast extract (which along with the FAN derived from the wheat gave a total FAN value of 859 mg N per liter of mash) resulted in the complete removal of the sugar in less than 72 hours! In fact, the sugar uptake may have been completed by 55 h if one assumed that the initial rate of uptake observed during the first 48 h had continued. A total of 629 mg FAN was taken up by the yeast when yeast extract was used as the supplement. In the unsupplemented mash, 73 mg FAN (75% of the available) was assimilated. In contrast, the amount of FAN taken up by the yeast from the mash supplemented with glycine (0.8 % w/w) was considerable (943 mg N per liter taken up in 120 h). However, the large uptake of FAN in the form of glycine did not result in a corresponding increase in the rate of attenuation (Fig. 4) or in increased cell proliferation (Fig.6). In fact the rates of attenuation and cell multiplication were slower in glycine-supplemented mashes than in the unsupplemented controls where the magnitude of the FAN uptake was only 7.7% of that observed with glycine. The results clearly indicated that glycine slowed down the fermentation and resulted in a lower yield of ethanol than found in the unsupplemented control (Fig.5).

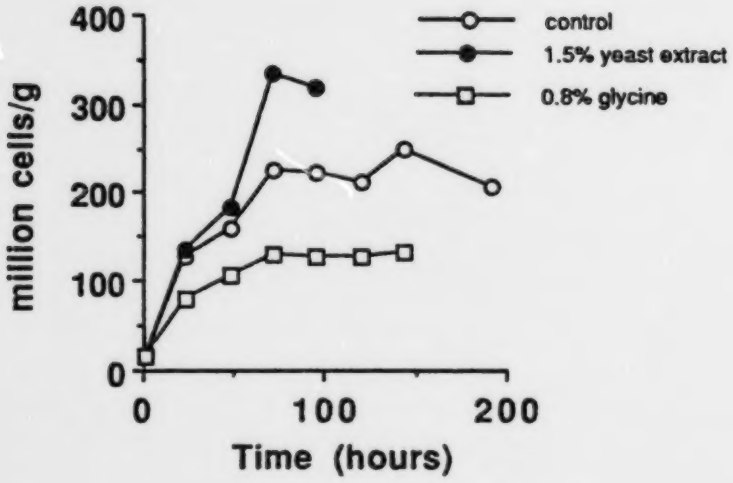


FIG. 6. Changes in the number of yeast cells during the fermentation of wheat mashes supplemented with yeast extract or glycine.

II. Relationship between free amino acid composition and fermentability of wheat mash.

The concentrations of free amino acids in wheat mash are appreciably different from the amino acid composition of hydrolyzed wheat mash proteins (Table 2). The amino acid composition of hydrolyzed and non hydrolyzed samples from the same wheat differed significantly in three respects. First, the *relative* amount of free arginine in the mash was more than twice the amount that would be available if all the proteins and peptides were hydrolyzed. Second, the amount of free glycine was only 38% of that found in hydrolyzed sample. Third, the most abundant free amino acid in the wheat mash was aspartic acid, while in the hydrolyzed sample it was glutamic acid. Glutamic acid is also the most abundant amino acid in wheat proteins . These differences in the amino acid compositions have important implications with regard to the suitability of mash to support yeast growth and stimulate (or inhibit) fermentation. These aspects are discussed in the following sections.

Table 2. Amino acid composition of wheat mash.

amino acid	mole % of free amino acids	mole % of amino acids after hydrolysis
lysine	3.2	3.9
histidine	1.2	2.1
arginine	9.5	4.3
aspartic acid	20.3	13.9
threonine	1.9	3.7
serine	8.2	6.3
glutamic acid	18.8	22.8
proline	5.5	7.8
glycine	3.9	10.4
alanine	10.9	8.8
valine	4.4	4.8
methionine	0.8	1.0
isoleucine	1.8	2.8
leucine	3.2	5.3
tyrosine	4.2	--
phenylalanine	2.1	2.2

The suitability of an amino acid to serve as a source of nitrogen was found to vary with the nature of the amino acid. For example glycine inhibited yeast growth and prolonged the fermentation time as shown in the previous section. Very little is known how other exogenously supplied amino acids or inorganic nitrogen sources such as ammonium ion or urea affect yeast growth and rate of fermentation in wheat mash. To study these aspects, various nitrogenous supplements were added to the wheat mash. Sufficient amounts of amino acids were used as supplements so that the concentration of α-amino nitrogen (not the total nitrogen) in the wheat mash was raised by 150 mg/liter. Thus the same mole percentage of glutamine, asparagine, lysine, arginine or histidine was added to the wheat mash, although the total nitrogen obtainable from each of these amino acids was different. Ammonium ion (in the form of ammonium sulfate) or urea was also added to raise assimilable nitrogen content by 150 mg per liter. Yeast extract added at 0.3% w/v increased FAN concentration approximately by 150 mg per liter. When mixtures of nitrogen compounds were used, each component contributed 150 mg N per liter.

Supplementing wheat mashes with various sources of assimilable nitrogen reduced the fermentation time except when the supplements were lysine or glycine

(Table 3). The time required to complete the fermentation at 20°C of the unsupplemented mash usually varied from 116 to 127 h although in certain experiments fermentation was completed within 100 hours. A fast fermentation invariably corresponded with high initial FAN content in the mash. The exact time at which the fermentation ended was estimated assuming that the fermentation continued at the rate observed during the last interval. The results presented in Table 3 are pooled from several experiments. The dissolved solid contents of wheat mashes in these experiments varied from 190 to 200 g per liter. The results are, therefore, normalized by converting the fermentation time of the control in each experiment to an arbitrary value of 100 h and by expressing the individual fermentation times in relative terms.

The effectiveness of a nitrogen source in stimulating fermentation varied with its nature and also with the amount of nitrogen that could be derived from that nitrogen source. Among the various compounds tested, arginine appeared to be the most effective nitrogen source in stimulating the fermentation of wheat mash. Yeast extract was also quite effective in promoting growth of the yeast and stimulating fermentation. The stimulatory effect of arginine may be at least partly through providing extra nitrogen. On an equimolar basis arginine provides four times as much nitrogen as an amino acid with a single amino group. The observed high rate of fermentation of wheat mashes supplemented with arginine may be therefore related to the availability of greater amounts of nitrogen than when equimolar amounts of any other amino acid was used as the nitrogen supplement.

Supplementing the wheat mash with glycine or lysine increased the fermentation time significantly. Both these amino acids inhibited growth of the yeast. Although the inhibitory effect of glycine was negligible at low concentrations, the rate of fermentation decreased significantly at 10.7 mM. This decrease in the rate of fermentation apparently was mediated by a low level of cell multiplication. The effect of lysine on wheat mash fermentation is far more severe than that observed with glycine. Lysine at a concentration of 10.7 mM increased the fermentation time by about 50% (Table 3) and this inhibitory effect appeared to be concentration dependent (Fig. 7A). As the concentration of lysine in the medium was raised from 10.7 mM to 32.1mM (equal to raising the α-amino nitrogen from 150 mg to 450 mg), the time required to complete the fermentation also increased.

Table 3. Effect of supplementing wheat mashes with various nitrogen sources on time required to complete fermentation by *Sacchromyces cerevisiae* [a]

Additive	Relative time of fermentation (hours)	Initial FAN (mg N/L)	Final FAN (mg N/L)
Arginine	43	246	18
Yeast extract	47	278	23
Leucine	58	276	16
Valine	62	264	18
Phenylalanine	62	215	19
Aspartic acid	64	246	18
Threonine	63	207	18
Glutamic acid	64	246	18
Ammonia	68	194	18
Serine	71	219	18
Cysteine	71	279	46
Urea	72	ND	19
Methionine	72	239	19
Tyrosine	73	244	18
Alanine	82	239	18
Isoleucine	84	182	18
Histidine	86	178	18
Control(no addition)	100	76	17
Glycine	120	228	19
Lysine	152	238	106

[a] Each amino acid was added at a concentration of 10.7 mM. Yeast extract at a concentration of 0.3% (w/v) provided about 150 mg FAN per liter of mash. Ammoniun sulfate and urea were each added at 5.4 mM. Wheat mashes initially contained 19 to 21 g dissolved solids per 100 ml.

The inhibitory effect of lysine, however, appeared to reach a saturation level at a concentration of 32.1mM. Increasing the lysine concentration further to 42.8 mM had virtually the same effect on the rate of fermentation as 32.1 mM. All the points in Figure 7A corresponding to concentrations of 32.1 mM and 42.8 mM overlap and therefore are indistinguishable.

It is quite apparent that the inhibitory effect of lysine on fermentation is mediated through inhibition of cell proliferation (Fig. 7B). While the number of yeast cells in the control increased and reached a maximum of 173 million cells per ml, the maximum cell number in the lysine supplemented media did not exceed 72.5 million cells per ml. Cell numbers were estimated by the direct microscopic method and included both viable and nonviable cells. On supplementing the wheat mash with lysine a high

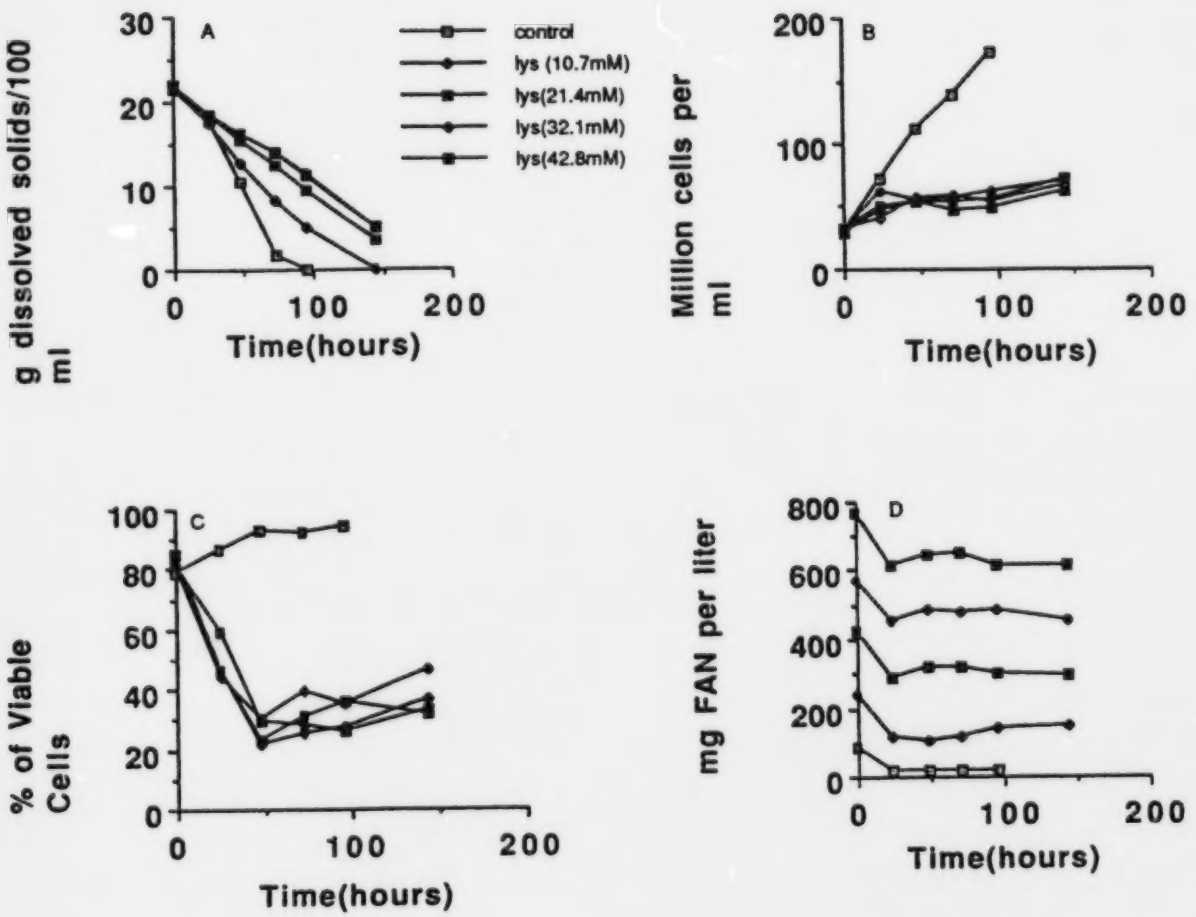


FIG. 7. Effect of various concentrations of lysine on the rate of fermentation, cell multiplication, % of cell viability and rate of FAN uptake. Symbols are the same in all figures as shown in Fig.7A.

proportion of yeast cells lost viability as evident from the inability of yeast cells to exclude methylene blue. This is in direct contrast to the effect of glycine on yeast metabolism. Glycine like lysine inhibited yeast growth and fermentation but did not affect the yeast viability (data not shown). After the initial loss of viability in lysine supplemented samples, there was modest recovery toward the end of fermentation (Fig. 7C). The active dry yeast used as the inoculum had a viability of 85%. In the control the viability increased to 95% during the course of the fermentation. Although all fermentors were inoculated with 30 million cells per ml (25.5 million viable cells per ml) lysine-supplemented samples at 48 h contained only 14 to 22 million viable cells per ml. During the same period the number of viable cells in the control increased to 105 million cells per ml. Thus the low rate of fermentation in lysine-supplemented mashes may be related to a partial loss of viability of yeast cells.

Most of the FAN in the mash was taken up by the yeast during the early part of the fermentation (Fig. 7D). All but 17 mg of FAN was taken up during the first 24 h from the control which initially contained 76 mg FAN per liter. The residual FAN which was not taken up most likely consisted of large peptides and soluble proteins. Irrespective of the level of supplement, about the same amount of FAN (130 to 140 mg FAN per liter) was taken up by the yeast when lysine was the nitrogen supplement. In these fermentors, considerable amounts of FAN remained at the end of fermentation. With other amino acids as nitrogen supplement, the amount of FAN that remained in the fermentor was the same as observed with the control (Table 3).

The inhibitory effect of lysine on yeast growth and fermentation could be relieved by adding other nitrogen sources to the wheat mash. We have not tested all amino acids with regard to their effectiveness in relieving the inhibitory effect of lysine but arginine, asparagine, glutamine, methionine, histidine, glycine, urea, ammonia and yeast extract were all found to reduce or completely eliminate the inhibitory effects of lysine: (Table 4). Amino acids were used at a concentration of 10.7 mM and urea at 5.35 mM. Ammonia was provided in the form of ammonium sulfate (5.35 mM) and yeast extract at 0.3% (w/v) yielded about 150 mg FAN per liter of mash (equal to the amount of FAN supplied by an amino acid with a single amino group and present at a concentration of 10.7 mM). One interesting observation is that although glycine and lysine when added singly to wheat mash inhibited yeast growth, the inhibition is reduced and not compounded when these two amino acids were added together. We did not test mixtures of pure amino acids for their effectiveness in relieving lysine induced inhibition of fermentation. The observation that yeast extract could counteract

the effects of lysine, however, suggests that mixtures of amino acids when present in the right proportion may relieve the inhibition induced by lysine.

Table 4. The ability of nitrogen sources to relieve lysine-induced inhibition of the growth of *Saccharomyces cerevisiae*. [a]

Addition	Relative time of fermentation (hours)	Final FAN (mg N per liter)
control(no addition)	100	17
lysine	152	106
Lysine+arginine	38	28
lysine+urea	45	48
lysine+asparagine	45	86
Lysine+glutamine	55	67
lysine+yeast extract	61	11
lysine+methionine	72	30
lysine+ammonia	89	124
lysine+histidine	109	188
lysine+glycine	116	229

[a]Unsupplemented wheat mash contained 76 mg FAN per liter. Each amino acid was added at a concentration of 10.7 mM. Urea and ammonium sulfate were provided at a concentration of 5.4 mM. Yeast extract at a concentration of 0.3 % (w/v) provided about 150 mg FAN per liter.

The amount of FAN left in the medium at the end of the fermentation varied with the nature of nitrogen source used to relieve the inhibition caused by lysine. We have consistently observed that between 20 and 25% of the total FAN in wheat mash is not usable. As all amino acids (with the exception of lysine and to some extent cysteine, see Table 3) are completely taken up by the yeast, the residual amount remaining at the end of fermentation is the non-usable fraction of FAN in wheat mash. When lysine was the sole nitrogen supplement, 44.5% of total FAN was not used by the yeast (Table 4). Assuming that the extent of uptake of FAN derived from wheat mash was the same as in the control, it can be calculated that only about 50% of the added lysine was taken up. Nitrogen compounds which relieved lysine-induced inhibition had different effects on lysine uptake (Fig. 8). The amino acids arginine and methionine promoted uptake of lysine to such an extent that virtually all of the added lysine was taken up (Fig. 8A). The effect of urea on the uptake of lysine was similar to those of arginine and methionine (Fig. 8B). Asparagine, glutamine, histidine, glycine, ammonia

and yeast extract partially relieved the lysine-induced inhibition of yeast growth, but did not promote complete uptake of FAN. In fact large amounts of FAN remained at the end of fermentation when histidine or glycine was added to counteract the effects of lysine (Fig.8B and Table 4).

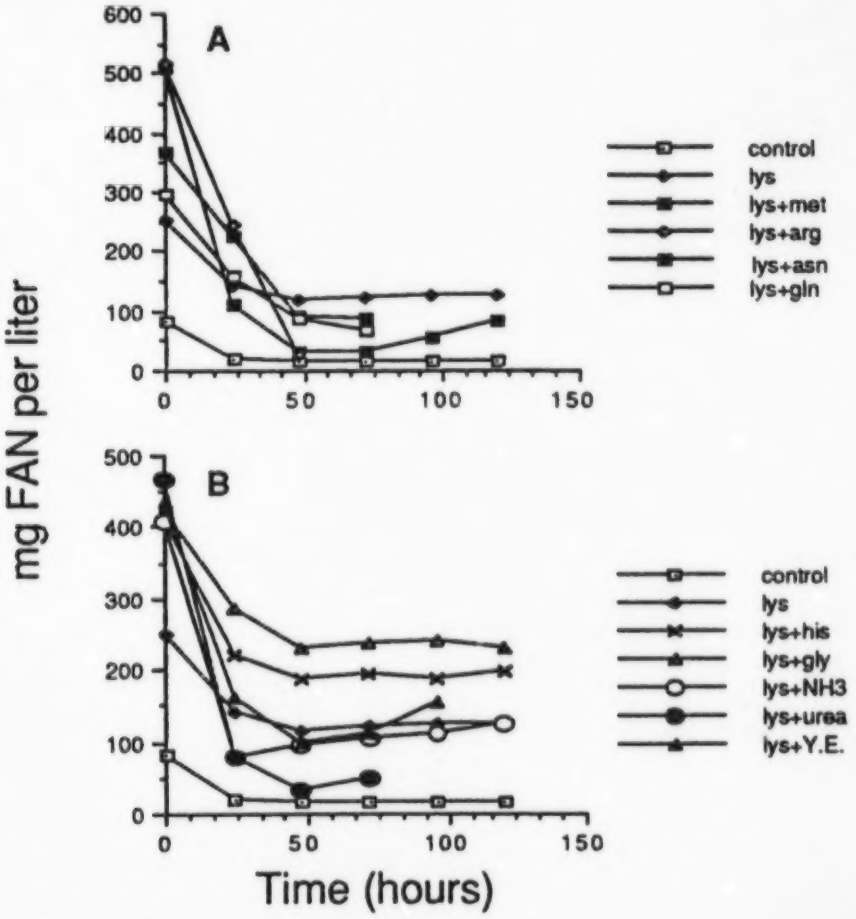


FIG.8.The effect of other nitrogen sources in relieving lysine-induced inhibition of sugar fermentation in wheat mashes with added lysine: changes in FAN values

The rate of fermentation was greatly influenced by the type of amino acid or other nitrogen source that was added to relieve the lysine-induced inhibition (Fig. 9). Arginine and urea were the most effective nitrogen sources, followed in order by asparagine, glutamine, methionine, yeast extract, ammonia, histidine and glycine. Although methionine promoted uptake of lysine to the same extent as arginine and urea (Fig. 8), the rate of fermentation was not stimulated to the same degree (Fig. 9).

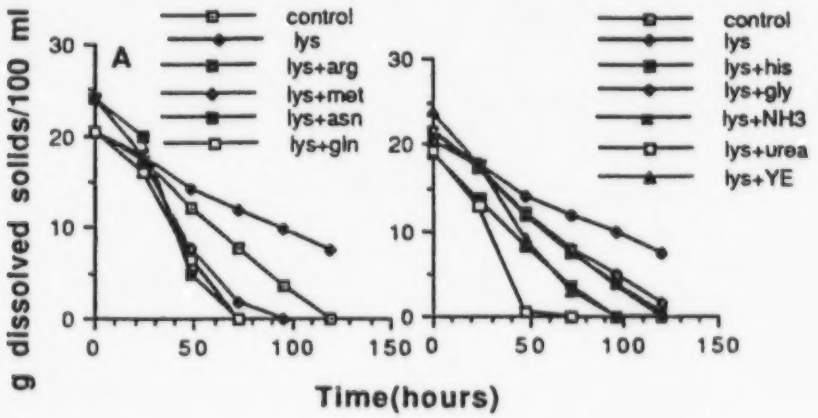


FIG.9.The effect of other nitrogen sources in relieving lysine-induced inhibition of sugar fermentation in wheat mashes with added lysine: changes in dissolved solids with time.

Since inhibition of fermentation by lysine is mediated through reduced cell proliferation and loss of viability (Fig. 7) it is to be expected that any compound that relieves this inhibition would prevent loss of viability and increase rate of cell multiplication. This was confirmed by the observation that rate of cell multiplication and maximum cell number attained were the greatest when arginine or urea was added to the wheat mash (Fig. 10). Asparagine also allowed rapid rate of cell multiplication, but maximum cell number attained was significantly less than that observed with either arginine or urea. All compounds that stimulated the rate fermentation had also caused increased cell multiplication.

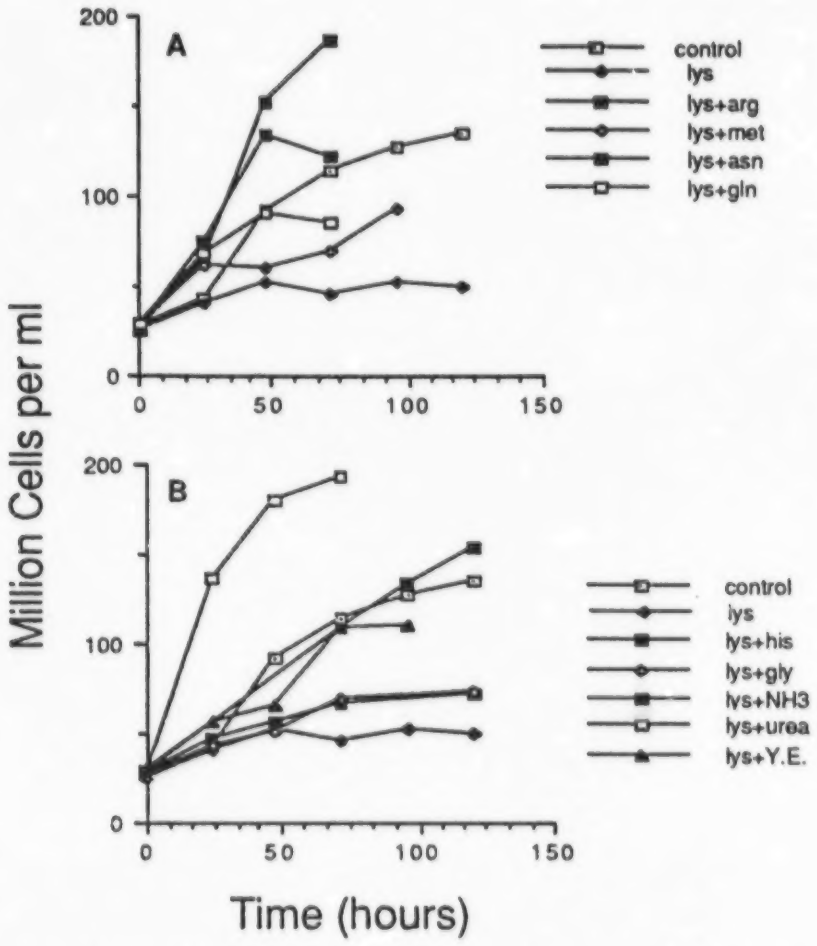


FIG. 10. The effect of other nitrogen sources in relieving lysine-induced inhibition of sugar fermentation n wheat mashes with added lysine: changes in total cell number

Compounds that relieved the inhibitory effect of lysine also prevented the lysine-induced loss of viability of yeast cells (Fig. 11). When lysine was the only nutrient supplement added to the wheat mash, the viability of the yeast population decreased by 80%. Addition of arginine and urea prevented this loss of viability and the percentage of viable cells in the population was restored almost to the same level as in the control. In general the nitrogenous compounds studied here may be classified into four groups according to their relative effectiveness in decreasing the lysine-induced loss of viability of yeast cells. The groups may be classified in the order of their effectiveness as follows; arginine, urea > asparagine, glutamine > methionine > yeast extract, ammonia, histidine, glycine.

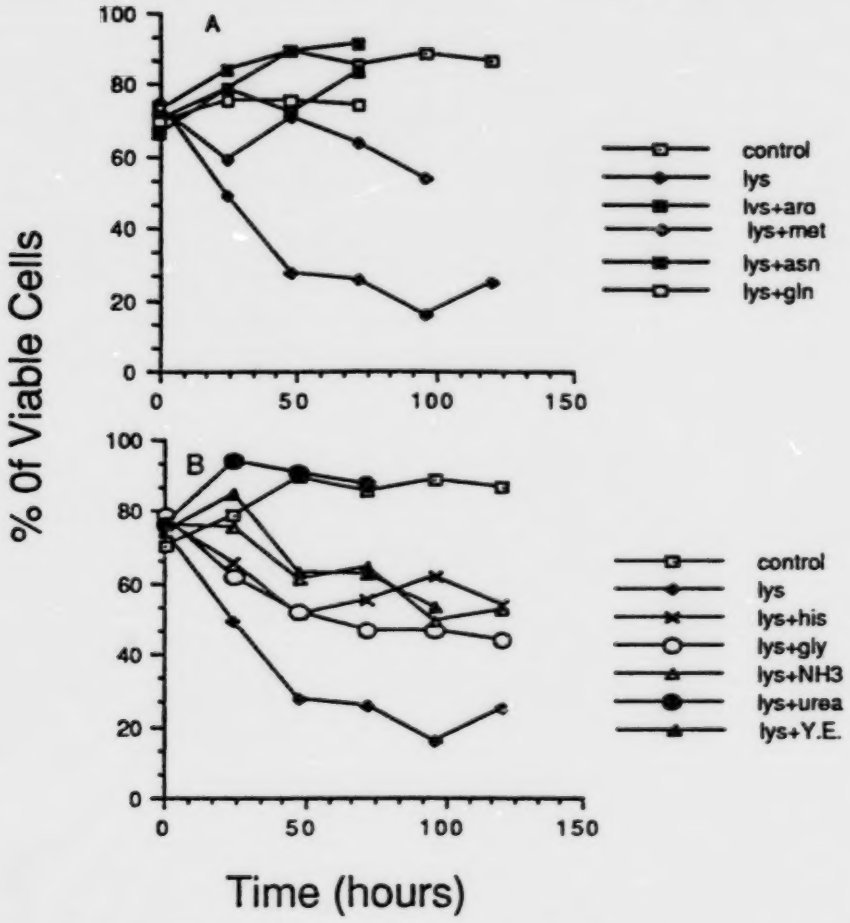


FIG. 11. The effect of other nitrogen sources in relieving lysine-induced inhibition of sugar fermentation in wheat mashes with added lysine: changes in the viability of cell population.

Addition of lysine to wheat mash not only decreased the rate but also affected the final ethanol yield (Fig 12). A significant amount of fermentable sugar remained unused in these mashes at the end of fermentation. All compounds which relieved the lysine induced inhibition of yeast growth increased the final ethanol yield although the time taken to complete the fermentation varied with the nature of the nitrogen source.

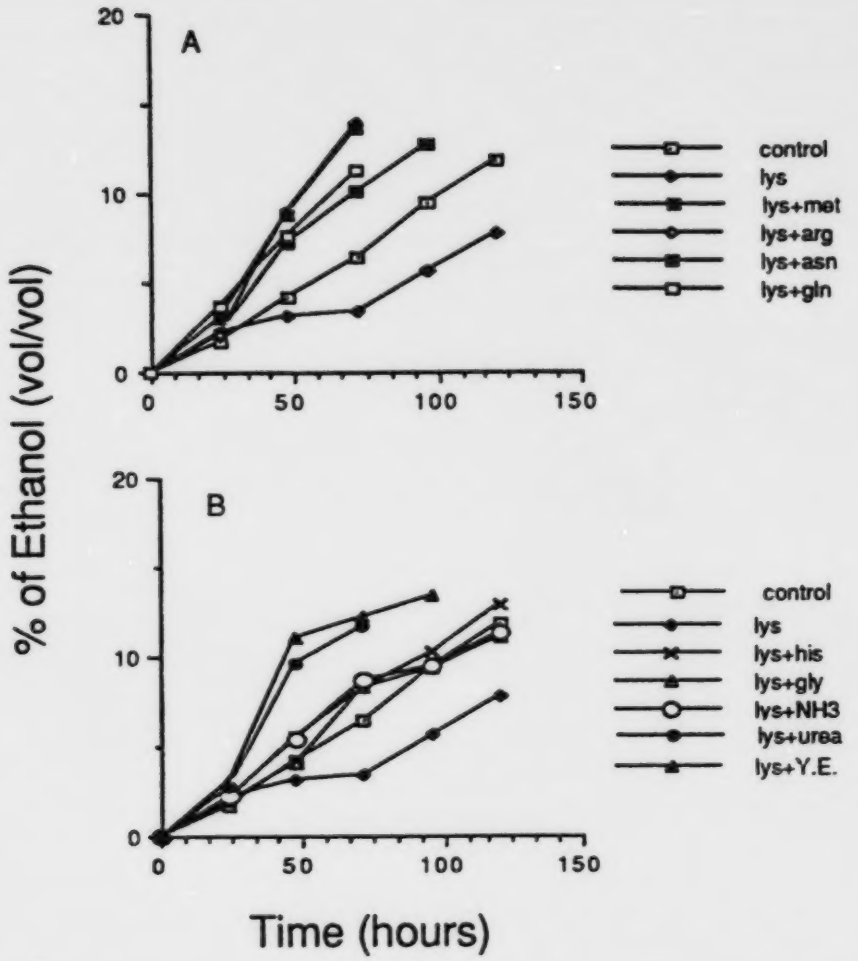


FIG. 12. The effect of other nitrogen sources in relieving lysine-induced inhibition of sugar fermentation in wheat mashes with added lysine: production of ethanol.

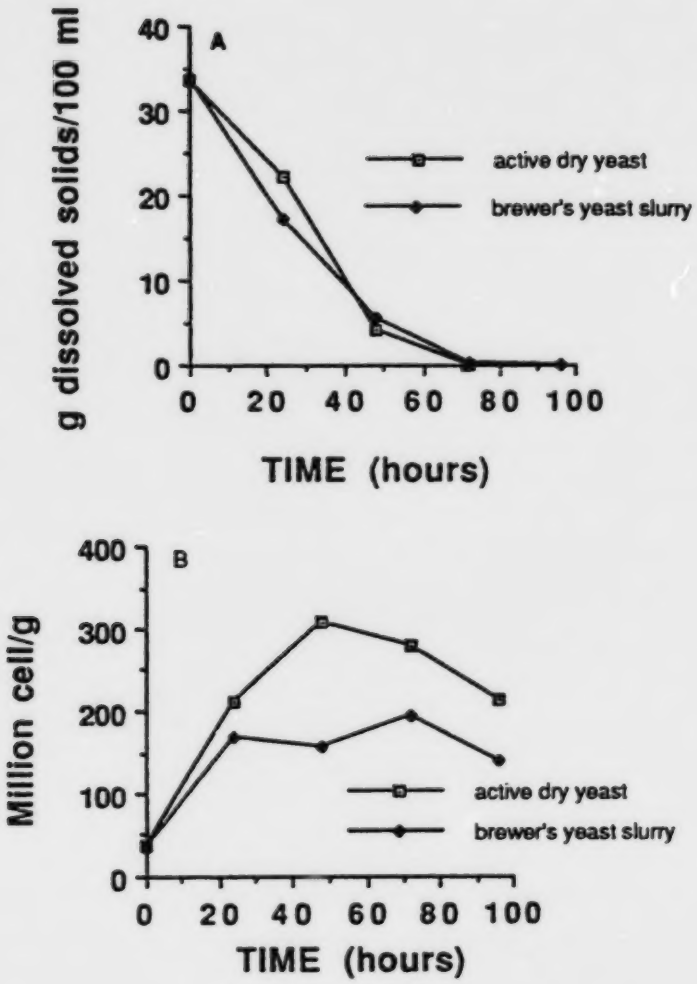


FIG. 13. Changes in (A) dissolved solid contents (B) total cell numbers during the fermentation of very high gravity wheat mashes by active dry yeast and brewer's slurry yeast. Yeast extract (1.5%) was added at the beginning of the fermentation.

III. Comparison of active dry yeast with brewer's slurry yeast for fuel alcohol production

All fermentations reported so far were done with active dry yeast. The possibility of using brewer's slurry yeast for the production of fuel alcohol by high gravity fermentation was investigated. A sample of brewer's slurry yeast was obtained from a local brewery. The cells were enumerated and used for the fermentation of a wheat mash which contained 30 g dissolved solids per 100 ml. Both yeasts were pitched at the same rate (37 million cells/g). The fermentation medium was supplemented with 1.5% yeast extract. The brewer's yeast cells were larger in size than active dry yeast, but both yeasts fermented the wheat mash at 20°C at about the same rate (Fig. 13A). The maximum cell number attained, however, was significantly higher with active dry yeast than with the larger slurry yeast cells (Fig.13B). With both yeasts, the conversion of sugars to alcohol approached the theoretical maximum. With active dry yeast it was 18.7% (v/v) and with brewer's yeast slurry an ethanol yield of 19% was obtained. In both cases, the fermentation was completed within 72 hours at 20°C although nutrient supplement in the form of yeast extract was required.

IV. Relationship between fermentation temperature and rate of fermentation

All fermentations reported so far were carried out at 20°C, although under industrial conditions of fuel alcohol production a temperature of 30°C is usually used. At higher growth temperatures the metabolic activities of the yeast increase and the fermentations are completed in shorter time than would be possible at low temperatures. High growth temperature, however, has its disadvantages. The amount of carbohydrates that can be fermented at elevated growth temperatures is limited because of a deleterious osmotic effect coupled with increased toxicity of ethanol on yeast growth and metabolism. The use of high fermentation temperature (30°C) is possible under industrial conditions because sugar concentrations are rarely higher than 20°P. It is not known whether or not very high gravity fermentation can be carried out at temperatures above 20°C.

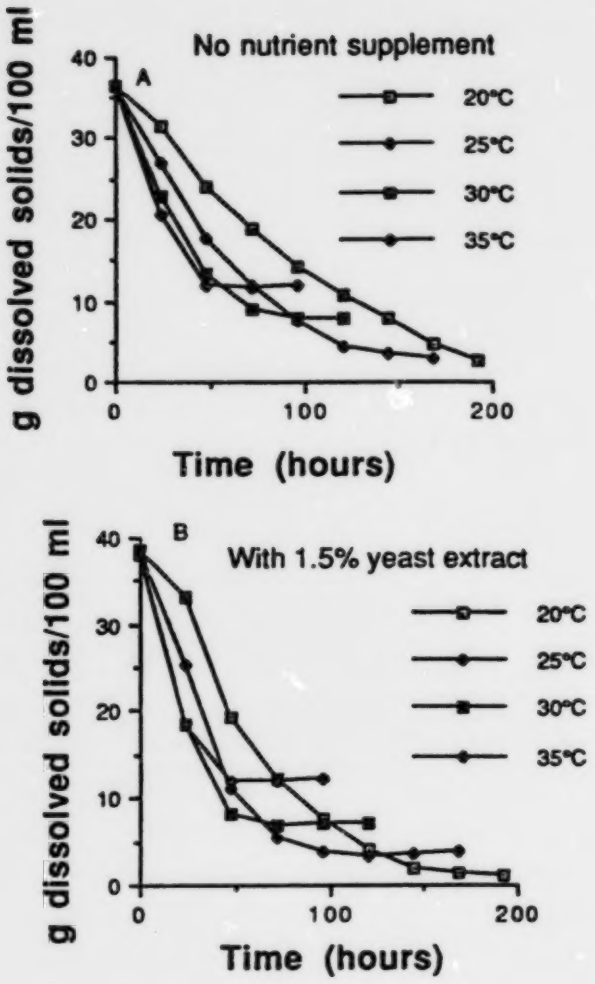


FIG. 14. Changes in dissolved solid contents during the course of fermentation of very high gravity wheat mashes at various temperatures and in the absence(A) or presence of (B) of added yeast extract.

In these experiments, wheat mashes containing 37 to 38 g of dissolved solids per 100 ml were fermented at 20, 25, 30 and 35°C. The effects of supplementing the mashes with 1.5% (w/w) yeast extract on the rates of fermentation was also studied. It is clear from Fig. 14A and 14B that at 20°C most of the sugar was fermented although the duration of the fermentation exceeded eight days. Supplementing the mash with yeast extract reduced the fermentation time by 3.5 to 4 days at this temperature. These observations are in agreement with the results reported earlier. At 25°C without any

nutrient supplement about the same amount of sugar was fermented, but in considerably less time (6 days). Although the initial rate of fermentation was faster with yeast extract supplement, the total time taken to complete the fermentation was not appreciably different from the unsupplemented mash at this temperature. At 30 and 35°C fermentations stopped long before the exhaustion of sugars. The duration of fermentation at 30°C and 35°C were 72 hours and 48 hours respectively. About 8 to 9 g of dissolved solids per 100ml remained unutilized at 30°C while at 35°C the unused amount was even greater (11 g/ 100 ml). One interesting and important observation is that supplementing wheat mashes with yeast extract did not affect the rates of fermentation or the final yields of ethanol at 30 and 35°C. This is in contrast to the observation that yeast extract had a significant positive effect on the rate of fermentation at 20°C.

While exploring the causes for the apparent lack of effect of yeast extract on the rate of fermentation at 30 and 35°C, we made a few interesting observations which may have important practical applications. At all temperatures there was a greater loss of yeast cell viability during the later stages of fermentation if the mash was supplemented with yeast extract. (Fig 15). With increasing fermentation temperature the loss of viability increased. At 30 and 35°C, nearly all yeast cells in mashes supplemented with yeast extract had lost viability by 96 h. At 25°C the same thing occurred only by 168 h. At 20°C, loss of viability was only partial and even after 192 h 40% of the cells remained viable in the mash supplemented with yeast extract. In the absence of any nutrient supplement the loss of viability was even less at this temperature.

The cause(s) for this loss of viability is not known. It is apparent that the cells did not lose viability as a result of the exhaustion of an energy source (fermentable sugar). As is evident in Fig. 14, significant amounts of sugars were present at 30 and 35°C while the yeast cells lost viability. Yeast extract stimulated the loss of cell viability and this stimulatory effect was further enhanced with increasing growth temperature. The cause for this stimulation is not clearly understood, but as the earlier sections in this report showed the presence of certain amino acids in excess or out of proportion with other amino acids could have caused the cells to lose viability.

The lack of effect of added yeast extract on fermentation at 30 and 35°C may be only apparent rather than real. At these temperatures enough yeast cells lost viability even in the absence of added yeast extract and it may be presumed that the cells that lost viability soon lysed and released nutrients into the medium. Thus the yeast extract

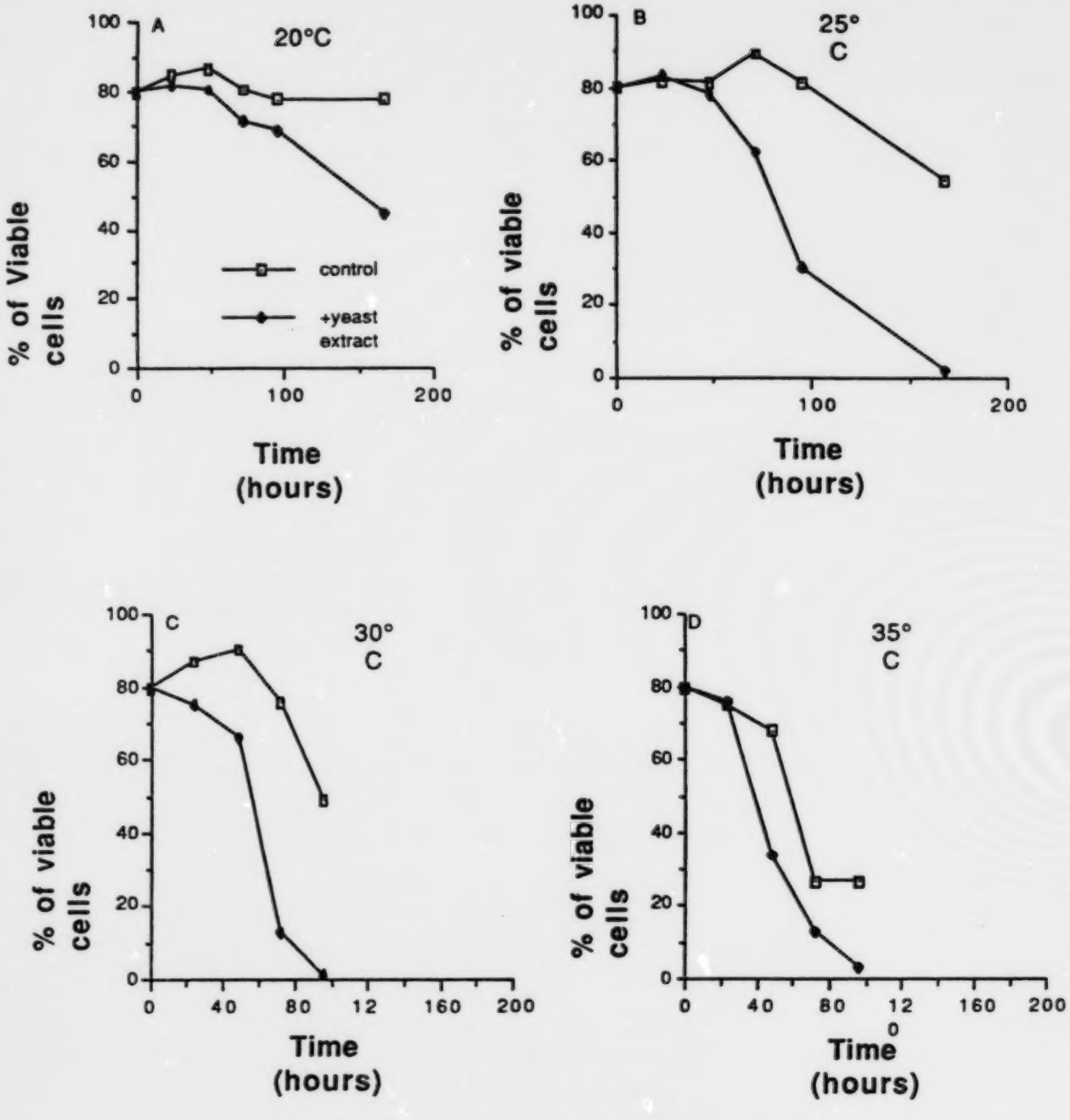


FIG. 15. Effect of fermentation temperature on the viability of yeast cells growing in wheat mashes in the presence or absence of added yeast extract.

supplemented and unsupplemented mashes may have sufficient amounts of nutrients available to stimulate fermentation. Estimation of free amino nitrogen during the course of fermentation showed that after an initial decline there was an increase in the FAN values at 30 and 35°C (Fig. 16). At 25°C the increase was only slight, while at 20°C there was no apparent increase. The greater loss of cell viability at 25, 30 and 35°C than at 20°C may be related to the availability of nutrients released through the lysis of cells.

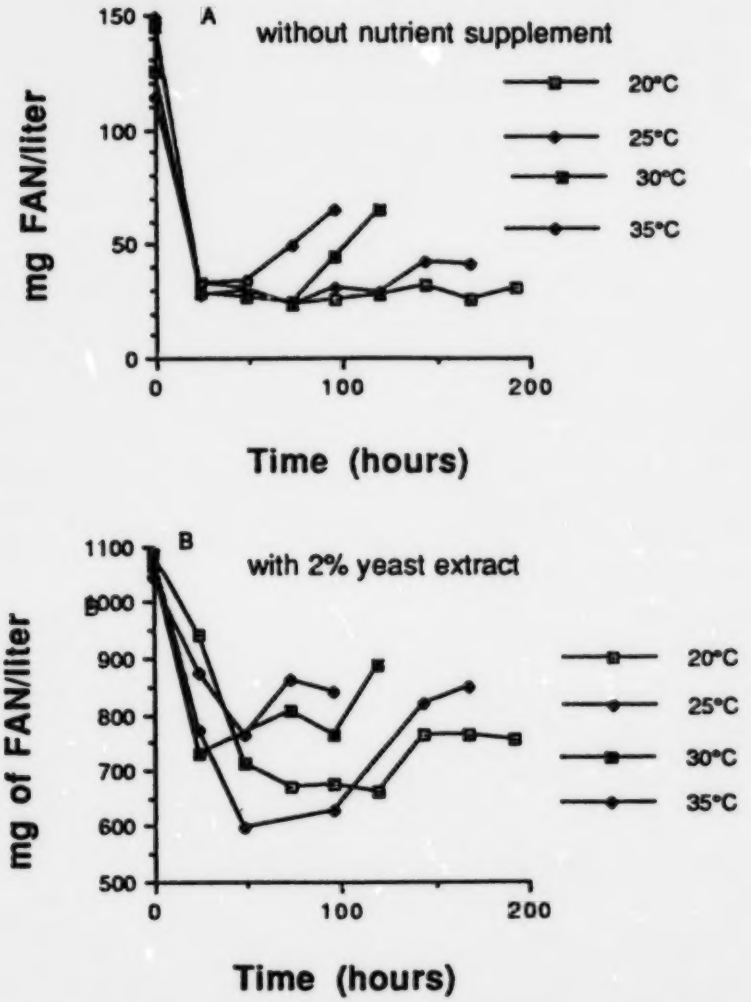


FIG.16. Changes in FAN values during the course of fermentation of very high gravity wheat mashes at various temperatures and in the absence(A) or presence of (B) of added yeast extract.

There is almost a linear relationship between the growth temperature and the maximum amount of sugar that can be fermented. (Table 5). Since some residual sugars are left at the end of fermentation at 25, 30 and 35°C, it can be assumed that under the fermentations conditions used here, maximum amounts of sugars have been fermented at these temperatures. At 20°C, however, the maximum value was probably even higher, since all the available carbohydrates were fermented.

The yield of ethanol at each fermentation temperature corresponded to the amount of sugars consumed. Supplementing wheat mashes with yeast extract yielded slightly more ethanol than in unsupplemented controls at 25, 30 and 35°C (Table 5). However, the difference between ethanol yields of supplemented and unsupplemented mashes was quite marked at 20°C. For example in the supplemented mash an ethanol yield of 20.5% (v/v) was obtained, but in the unsupplemented mash the final value was only 18.3% (v/v).

Table 5. Relationship between fermentation temperature, the quantity of carbohydrate consumed, and ethanol produced during very high gravity fermentation.

Fermentation	Maximum sugar consumed		Final ethanol yield (% v/v)	
°C	control	with 1.5% yeast extract	control	with 1.5% yeast extract
20	34.1	37.5	18.3	20.5
25	33.6	34.8	18.2	19.2
30	28.6	31.2	15.7	16.9
35	24.7	25.8	13.4	14.0

The highest amount of ethanol (20.5% V/V) was obtained at 20°C with nutrient supplement . Such a high yield of ethanol in such a short time (eight days) has not been reported previously. There is a possibility that at this temperature even greater yields of ethanol may be attainable. At higher temperatures the concentrations of ethanol obtained appear to be limited.

V. Effect of different pitching rate

Studies conducted in this laboratory have shown that high gravity worts could be fermented without significant proliferation of yeast cells if the pitching rate (level) was increased. This is one of the methods recommended to prevent sluggish or stuck fermentation. Sluggish or stuck fermentations result because of the inadequacy of nutrients required for the growth and multiplication of yeast cells. Although we never

experienced stuck fermentation with wheat mash, fermentation was somewhat slow because of low level of assimilable nitrogen present in wheat mashes. As we have shown in previous sections, fermentation could be accelerated by supplying an external source of assimilable nitrogen. An alternative is to use a high pitching rate so that cell proliferation is not mandatory for the fast rate of fermentation.

In the following set of experiments six pitching rates were studied. The wheat mashes were pitched at the rate of 15, 30, 45, 150, 300 or 750 million cells per g of mash. The three low levels corresponded to the pitching rates normally employed under high gravity and very high gravity fermentations. The recommended pitching rate is one million cells per ml per degree plato. The object of this study was to explore whether high pitching rates such as the ones used here (150, 300 or 750 million cells/ g mash) could accelerate fermentation without any nutrient supplement.

Mashes containing 37 to 42 g dissolved solids per 100 ml were used in these studies. Yeast extract (2.0% w/w) was added to some fermentors as nutrient supplement. The results showed that more than 168 hours were required to complete the fermentation if wheat mashes were pitched at 15 or 30 million cells per g and no nutrient supplement was provided (Fig 17A) At much higher pitching rates of 150 and 300 million cells per g the fermentations were completed by 168 and 120 hours respectively. Addition of yeast extract stimulated the rate fermentation at all pitching rates. (Fig 17B). Completion of fermentation is more or less independent of pitching rate if the mash is supplemented with yeast extract, although the rate of fermentation is still a function of the pitching rate. The differences in the time taken to complete the fermentation at different pitching rates however, became smaller in the presence of added yeast extract. For example, at pitching rates of 15 and 30 million cells the fermentation was completed within 120 h, while at higher pitching rates it took only 96 h. Thus with nutrient supplement, there is only marginal advantage in using high pitching rates.

FIG. 17.Changes in dissolved solid contents during the fermentation of very gravity mashes pitched at different rates and in the absence(A) or presence (B) of added yeast extract.

Table 6. Effect of different pitching rates on the rate of sugar consumption during very high gravity fermentation of wheat mash.

Pitching rate million cells /g		amount of sugar consumed (g/100 ml)[1] Total[2]	0 to 24 h	24 to 48 h
15	control	26.7 (>168)	3.2	6.9
	+ Y.E	35.4 (120)	2.9	19.1
30	control	29.5 (>168)	6.5	5.9
	+Y.E	37.2 (96)	5.3	13.0
150	control	34.2 (144)	7.5	9.4
	+Y.E	42.4 (96)	20.5	11.7
300	control	38.4 (120)	15.2	7.2
	+Y.E	40.1 (96)	20.8	9.8

[1] Dissolved solids were estimated from specific gravity measurements.

[2] Figures in parentheses indicate the number of hours required to complete the fermentation.

The greatest advantage of a high pitching rate appears to be that fermentation gets an early start. The advantages are two-fold. First, during the first 24 h, about five times as much sugar was consumed at a pitching rate of 300 million cells per g as when the pitching rate was only 15 million cells per g (Table 6). With added nutrient supplement the amount of sugar used was seven times greater at a high pitching rate than at a low pitching rate. The second advantage is that with a high pitching rate no lag was observed before the fermentation accelerated.

Our results showed that at all pitching rates both biomass and cell number increased (Figs. 18 & 19). At low pitching rates (15 or 30 million cells per g) the increase in biomass was considerably greater in yeast extract supplemented mashes than in controls. The total number of cells increased several fold at low pitching rates with and without yeast extract supplement. In these samples there was no appreciable loss of cell viability. At high pitching rates, the level of multiplication was small. At a

pitching rate of 300 million cells per g the cells multiplied 1.4 times in the absence of yeast extract supplement, while cell number increased 1.6 times in its presence.

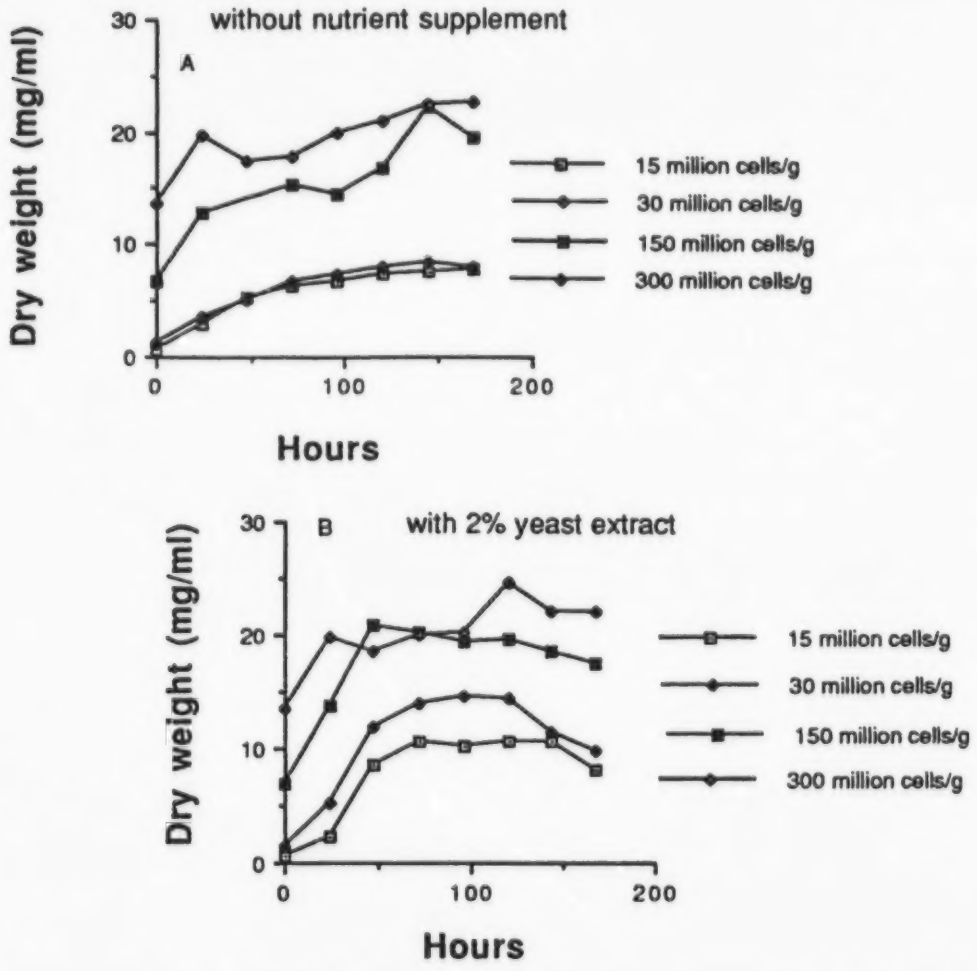


FIG. 18. Changes in biomass (dry weight) of yeast cells during the fermentation of very high gravity mashes pitched at different rates and in the absence(A) or presence (B) of added yeast extract.

Irrespective of the pitching rates about the same number of new cells were produced in each case. For example about 180 million new cells were produced in yeast extract supplemented mash which was pitched at a rate of 300 million cells per g. About the same number of new cells were formed in mash pitched at the rate of 15 million cells per g. The assumption that cells do not grow or multiply at very high pitching rates is not substantiated by our results. There was a small degree of cell

multiplication even when a wheat mash was pitched at a rate of 750 million cells per g. We have established that at high pitching rate a large proportion of the yeast cell population loses viability. Because of this loss of cell viability at high pitching rates, cell multiplication may go unnoticed especially if growth is monitored by viable plate count alone. The suggestion that cells do not grow or multiply and that all the sugars consumed under these circumstances are used for maintenance purpose is not valid at high pitching rates. As our results have shown, not only was there growth in terms of biomass increase, but there was actual cell proliferation.

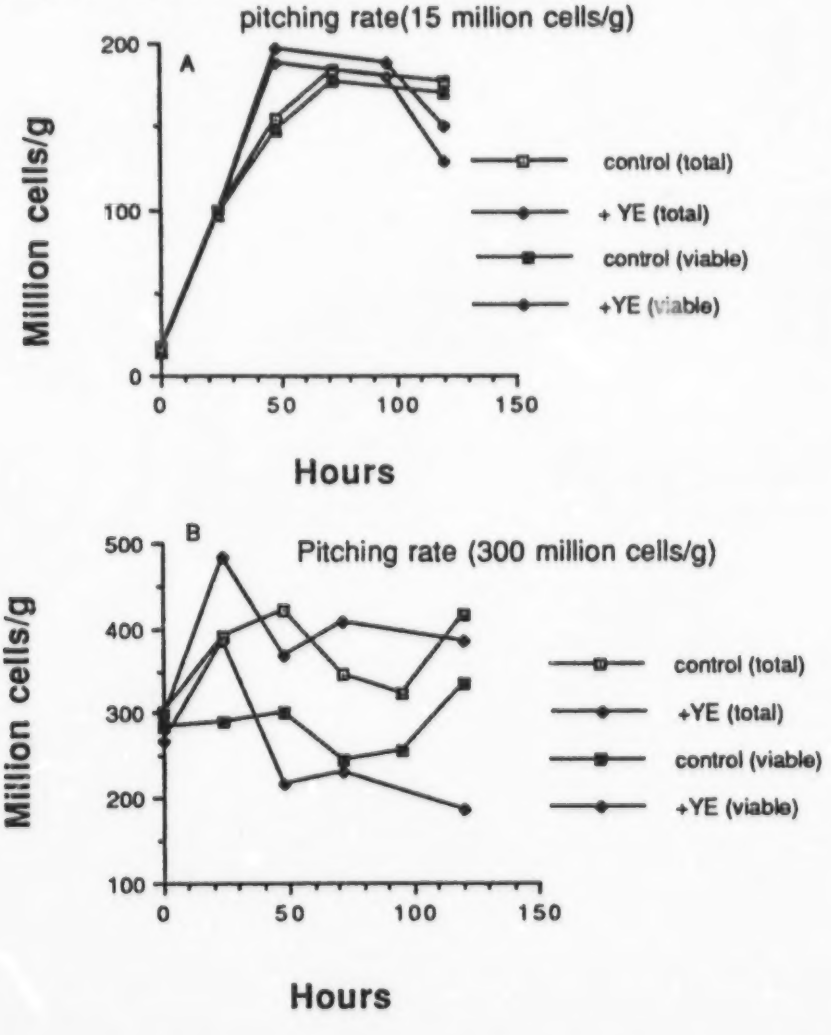


FIG. 19. Changes in total and viable cell numbers during the fermentation of very high gravity wheat mashes in the presence and absence of added yeast extract. The mashes were pitched at (A) 15 million cells/g and (B) 300 million cells/g.

Although about the same number of new cells were produced at low and high pitching rates the average size of the cells in the population was smaller at high than at low pitching rate (data not shown). Results obtained with biomass determination are in agreement with this observation. For example, at low pitching rates the biomass increased by 9.9 mg (dry wt) per ml while at high pitching rates the increase was only 6.3 mg / ml, although in both cases the number of newly formed cells were about the same. At low pitching rate the cell population had a normal distribution while most of the cells in samples pitched at high rate were small and the size distribution was skewed to the left.

Pitching very high gravity wheat mashes at high rates resulted not only in rapid completion of fermentation but also yielded greater amounts of ethanol (Table 7). At a pitching rate of 750 million cells per g of mash, an ethanol yield of 21.5% (v/v) was achieved. Assuming that all the sugars were monosaccharides it can be calculated that this concentration of ethanol is equal to a theoretical yield of 97.6%. At low pitching rates, ethanol yields were considerably less than the theoretical maximum. This suggested that greater amounts of sugars were channelled at low pitching rates for other cellular processes or for the synthesis of products other than ethanol.

Table 7. Relationship between pitching rates and ethanol yield during fermentation of very high gravity wheat mashes.

Pitching rate million cells/g mash	Amount of sugar consumed g reducing sugar/ 100 ml)	%ethanol v/v	% of theoretical yield
45	30.2	17.4	89.2
300	31.9	19.0	92.2
750	34.1	21.5	97.6

VI. Effect of high pitching rate on the fermentation of very high gravity mash at 35°C

All studies reported in the previous section on the effects of high pitching rates on the fermentation of very high gravity mashes were done at 20°C. Virtually nothing is known about the relationship between pitching rate and the fermentation temperature. In the following study very high gravity wheat mashes (37 g dissolved solids per 100 ml) were inoculated with 300 million yeast cells per g of mash and the

rate of fermentation at 35°C was followed. Fermentation was incomplete at this temperature both in the presence or absence of added yeast extract (Table 8). The amount of sugar consumed as well as the quantity of ethanol produced was considerably less than that observed at 20°C at equivalent pitching rates. Exogenously added yeast extract inhibited the fermentation to a small extent as indicated by the decreased yield of ethanol. The theoretical yield of ethanol in the presence of added yeast extract was considerably less than that observed in its absence. This suggested that the metabolism of yeast fermenting very high gravity mashes at 35°C in the presence of added yeast extract is altered, and more sugar is channelled for the production of products other than ethanol.

Table 8. Fermentation of very high gravity wheat mashes at 35°C with a pitching rate of 300 million cells per g mash.

Nutrient supplement	Amount of sugar consumed (g reducing sugar/100 ml)	% Ethanol (v/v)	% of theoretical yield
None (control)	17.5	10.6	93.7
Yeast extract (1.5%)	16.5	7.6	71.1

There was a rapid uptake of FAN by the yeast cells during the first 24 h. This was followed by a gradual release of FAN, with the effect that there was more FAN in the fermentation medium at the end of the fermentation than at the beginning. This was true with and without the addition of yeast extract, although greater amounts of FAN were released from yeast extract supplemented mash. By 48 hours over 80% of cells had lost viability under both treatments. The release of FAN during the fermentation corresponded to the loss of viability, an indication that the FAN was derived through the lysis of yeast cells.

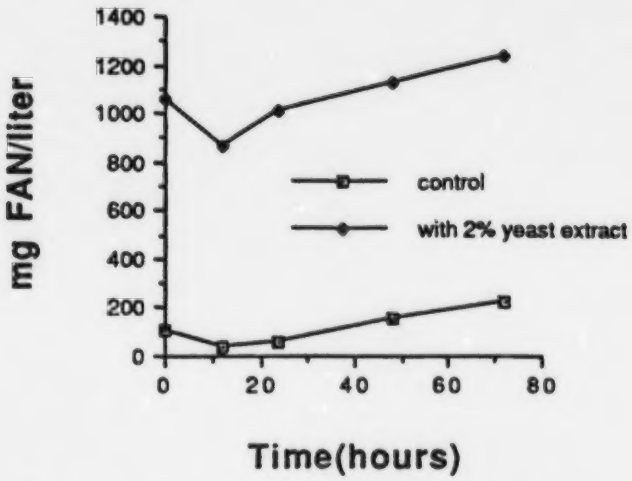


FIG. 20. Changes in FAN values of very high gravity wheat mashes during fermentation at 35°C with and without added yeast extract.

VII. Comparison of active dry yeast with a thermotolerant strain of *Saccharomyces diastaticus*

We have shown in the previous sections that the amount of sugar that can be fermented with active dry yeast which is normally used under industrial conditions steadily decreases with increasing fermentation temperatures. This decline results partly from the enhanced ethanol toxicity to yeast at increasing growth temperatures. The aim of this experiment was to evaluate whether high-temperature resistant strains of yeast can be used for the fermentation of very high gravity mashes. A highly flocculent strain of *Saccharomyces diastaticus* reported to grow and ferment well at 40°C, was obtained from the Labatt Brewing Company Limited (London, Ont.). This strain has been shown to ferment up to 20% sugar at 40°C yielding 11.5% (v/v) alcohol. This reported fermentation, however, was carried out not in grain mashes, but in rich microbiological media which contained in addition to sugar other nutrients such as peptone, yeast extract, ammonium sulfate and other minerals.

Our attempts to ferment very high gravity wheat mashes (>30 g disolved solids /100 ml) completely at 35°C with this strain of yeast were not successful. Addition of yeast extract to these mashes stimulated the sugar consumption, but did not result in increased ethanol yield. This suggests that at high fermentation temperature the metabolism of yeast in the presence of extra nutrients (yeast extract) changes and the efficiency of conversion of sugars to ethanol decreases. With yeast extract

supplement, 96% of the fermentation was completed within the first 24 h, while only 83% was completed during the same period in the absence of any added yeast extract.

VIII. Cascade fermentation and yeast recycling

In a cascading system of fermentation a number of fermentors are connected in series and fixed volumes are transferred in series (one fermentor to another) at regular intervals. The number of fermentors required would depend on the fraction of the fermentor liquid transferred at each interval and the duration of the interval. The cascading system of fermentation is the nearest thing to continuous fermentation and it allows the use of a number of small fermentors (previously used in batch processes). Cascade operation maintains high output. Another advantage is that there is no need to pitch every batch of mash with new lots of active dry yeast.

The same objectives described above can be achieved with a single fermentor if the residence time is long enough and when only a fraction of the contents of the fermentor is removed at regular intervals. This approach was taken in the following experiments to evaluate whether a cascading style of fermentation with recycling of yeast could be adapted to high gravity fermentation.

The study was conducted as a two-stage experiment. In the first stage wheat mashes (22 g dissolved solids per 100 ml) were inoculated with active dry yeast (27 million cells per ml) and fermented at 20°C. At 48 hours half of the well mixed contents of the fermentor was removed and replaced with an equal amount of fresh wheat mash. The cycle was repeated every 48 hours. Results presented in Fig. 21 show that from the second cycle onwards all fermentable carbohydrates in the fermentor were exhausted within the 48 h cycle. A steady state seemed to have attained with respect to the course of fermentation and the amount of alcohol porduced. Results for five cycles only are shown in Fig 21, but we have observed that the pattern of fermentation was repeated for many more cycles. Even after several recyclings, the yeast had not lost its vigor. The viability was maintained between 93 and 95%.

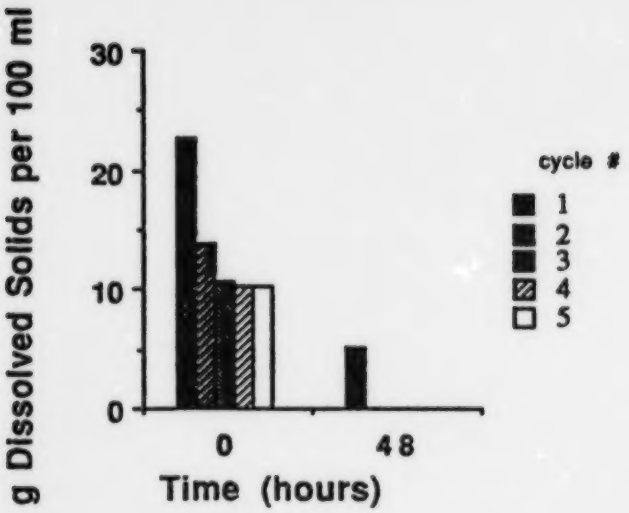


FIG. 21. Relationship between cascade fermentation and the amount of dissolved solids present at the beginning and end of cycle period (48 hours).

A steady state cell population of 290 to 295 million cells per ml was maintained (Fig 22). A final ethanol yield of 12.8 to 13.0% (v/v) was obtained (Fig. 23). Compared to an alcohol yield of 8 to 9 % (v/v) reported in a cascade operation in Tennessee, this is a significant improvement in concentration. In that process, however, a fermentation temperature of 31° C was used and the fermentation was completed within 24 hours. In the present study, a cycle time of 48 hours was arbitrarily chosen; all the carbohydrates in the fermentor were exhausted within 24 hours after the fermentation had reached a steady state. This indicates that the cascade system with a sugar concentration of 22 g per 100 ml mash is possible for production of 13% ethanol by volume within 24 hours.

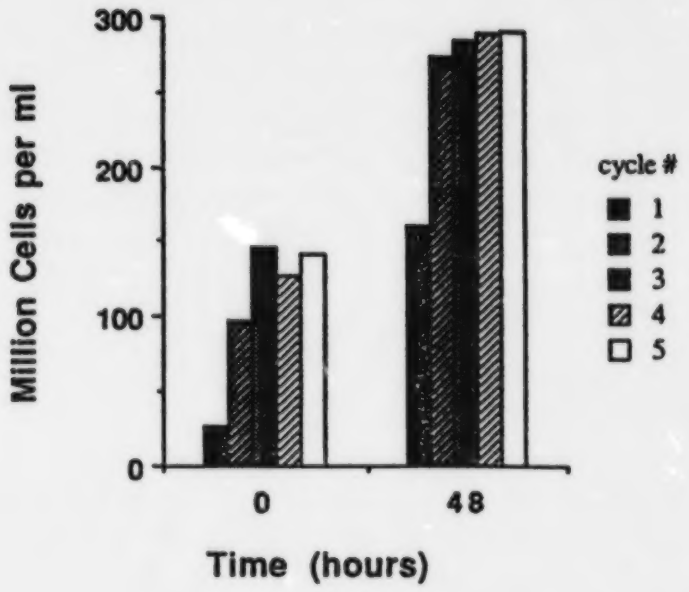


FIG.22. Relationship between cascade fermentation and the number of yeast cells present at the beginning and end of the 48 h cycle.

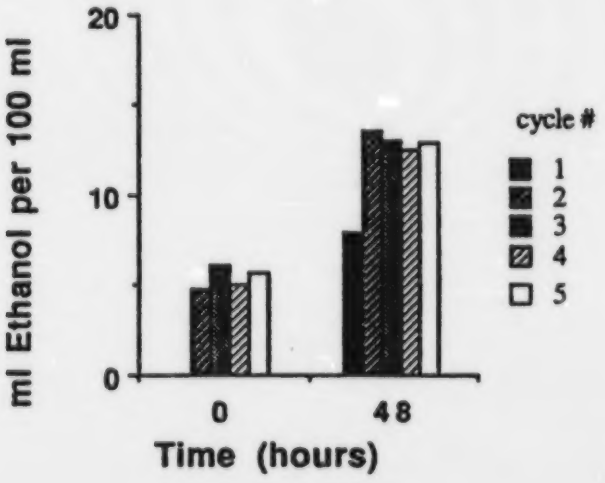


FIG.23 The concentation of ethanol at the beginning and end of the cycle during cascade fermentation.

During the second stage of the experiment, the dissolved solids in the fermentor were raised by adding fortified wheat mash (40 g dissolved solids per 100 ml) to a cascade system already in steady state. A wheat mash with 22 g dissolved solids per 100 ml was used for the cascade system. On addition of the fortified mash (fortified by adding freeze dried wheat hydrolysate to regular wheat mash), the dissolved solid content at the beginning of the cycle was raised to 20 g per 100 ml rather than the usual 11 g/ 100 ml observed when regular mash was added at 50%. Assuming that all of the carbohydrate was fermented the concentration of ethanol in the first cycle should correspond to the initial sugar concentration of 31g per 100 ml. This value would increase in the subsequent cycles if fortified mash was used to replenish the fermentor, and finally a steady state value would again be reached. The results presented in Fig. 24 show that the amount of carbohydrate fermented within the cycle period steadily decreased with an increasing number of cycles. There was a corresponding decrease in the cell number (Fig. 25) and alcohol yield (Fig.26). Increasing the cycle time to 72 hours was not sufficient to ensure complete utilization of sugars.

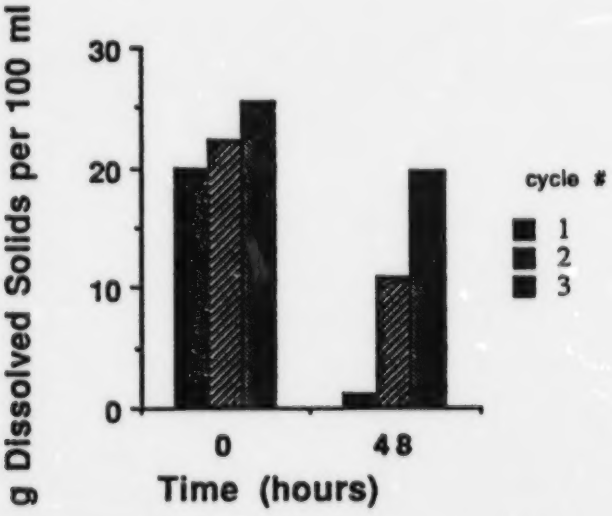


FIG.24 Effect of 48 h recyling of half of the contents of fermentor on the utilization of carbohydrate from very high gravity wheat mashes (>31 g dissolved solids per 100 ml)

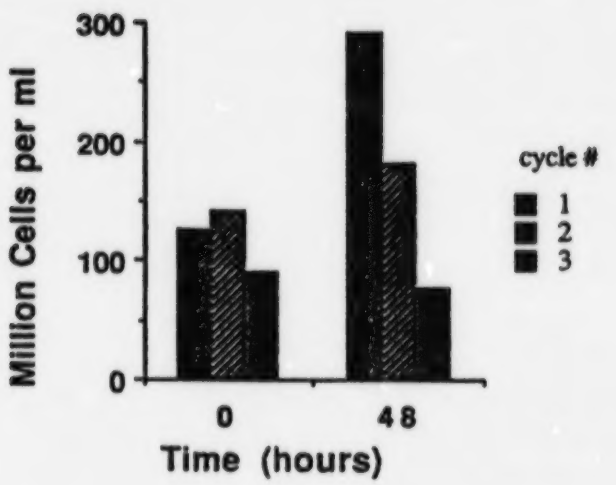


FIG. 25. Number of yeast cells present at the beginning and end of three cycles. The details are the same as described in Fig.24

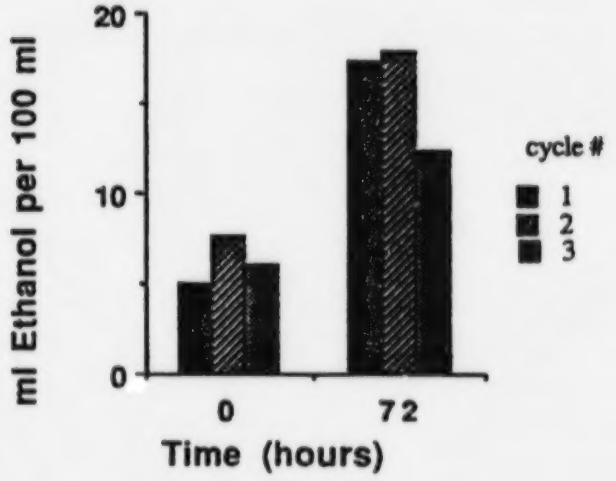


FIG. 26 Effect of 48 h recycling of the fermentor contents on ethanol yield. The details are the same as in Fig. 24.

For the successful operation of the cascade system of fermentation and recycling of yeast, a certain degree of cell multiplication is necessary. Any condition that retards or prevents cell multiplication would slow down or completely inhibit

fermentation. At high concentration of ethanol, yeast may stop growing and may even lose viability. The deleterious effects of alcohols is further compounded by the increased osmotic effect exerted by high concentrations of sugar in the mash. Under the conditions described here, a certain amount of alcohol is present at the beginning of each cycle. As the results presented show (Fig. 22) the growth of the yeast was not impaired by alcohol concentrations up to about 7%(v/v). The concentration of ethanol at the beginning of a cycle of the fermentation of fortified mash (>31 g dissolved solids per 100 ml) was considerably higher than this value. The reason for the incomplete fermentation of sugars in this case may be attributed to the negative effect of alcohol on cell multiplication.

IX. Incremental Feeding

It is a common practice to use incremental feeding when large amounts of substrates are to be converted to ethanol. In the sake fermentation, hydrolyzed rice starch is fed to fermentors at regular intervals over a number of days, and by this method an ethanol yield as high as 20% (v/v) could be achieved. Although we have been able to ferment very high gravity wheat mashes with a single zero time feeding to produce 21.5% (v/v) ethanol, incremental feeding might raise the ethanol yield further or reduce the fermentation time.

It was found however that the total time taken to complete the fermentation was independent of the method of feeding the substrate (Fig.27). With incremental feeding, the yeast multiplied to a greater extent than with single feeding (Fig.28) but this increased cell proliferation did not result in an earlier completion of fermentation. It appears that high concentrations of ethanol produced during the early stages of fermentation affected the yeast metabolism and slowed down the conversion of sugars to ethanol. The results suggest that yeast cells produced during incremental feeding (low sugar concentration during the cell multiplication stage) are more susceptible to the alcohol-induced inhibition than cells from single feeding fermentation.

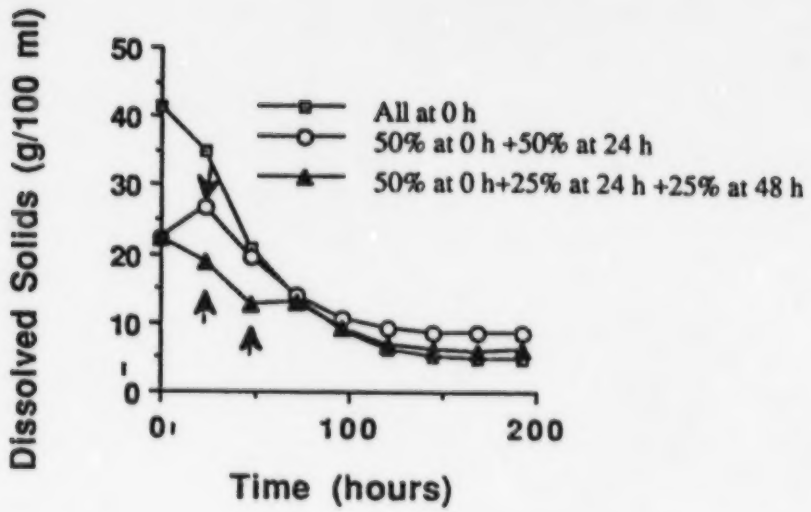


FIG. 27 Rate of disappearance of dissolved solids during fermentation of wheat mashes fed to fermentors at different times.

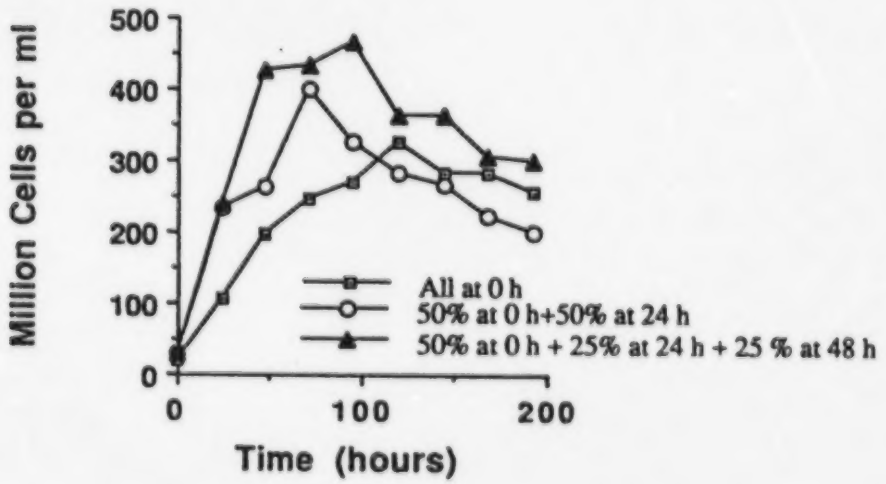


FIG. 28. Relationship between incremental feeding and cell multiplication in a very high gravity wheat mash.

Conclusions and Impact

1. Very high gravity (VHG) fermentation technology can be applied to the production of fuel alcohol from grains such as wheat. Sugar concentrations as high as 38 to 40 % (w/v) can be completely fermented, with ethanol yields as high as 21.5% (v/v).

2. The time required to produce such high concentrations of ethanol is relatively short (6 to 8 days) and this can be further shortened to 3-4 days by supplementing the fermentation medium with the extra nutrients required for yeast growth.

3. Wheat as a raw material for fuel alcohol production contains all the nutrients required for growth and multiplication of yeast. The amount of usable nitrogen, is low in wheat mash, although normally sufficient to allow the completion of fermentation. Fermentation can be accelerated by making more free amino nitrogen available for yeast growth either through in situ hydrolysis of wheat proteins with added proteolytic enzymes or by adding various sources of nitrogen (yeast extract, urea, ammonia or pure amino acids) to the mash.

4. The amino acids in wheat mashes are most efficiently used by the yeast, The reason for this appears to be the low lysine content of the wheat mashes. Low concentrations of lysine and glycine and high concentration of arginine maximize the efficiency of utilization of free amino nitrogen. Wheat mash appears to offer ideal growth (fermentation) conditions.

5. In the absence of any added nutrient, a very high pitching rate (>300 million cells per ml) accelerates fermentation of wheat mash. In the presence of nutrient supplement (1.5 to 2.0 % yeast extract) the pitching rate has very little effect on the rate of fermentation. The accelerating effect of the high pitching rate on fermentation in the absence of nutrient supplement appears to be through the release of nutrients from lysed yeast cells.

6. The amount of sugar that can be fermented is a function of the fermentation temperature. At 30°C, the maximum amount of sugar that can be fermented varies between 24 to 26 g per 100 ml with an ethanol yield of 15 to 16% (v/v).

The maximum yield of ethanol is even less at 35°C (13-14% v/v). The highest concentration of ethanol obtained at 20°C was 21.5% v/v. Use of a thermotolerant yeast (*Saccharomyces diastaticus*) did not improve the alcohol yield at 35°C.

7. High gravity fermentation can be applied by the cascade fermentation system which allows recycling of the yeast. The cycle can be repeated many times. However, fermentation is incomplete and the yeast loses viability rapidly if the sugar concentration in the wheat mash exceeds 30 g per 100 ml in the cascade system.

8. The total time taken or the final yield of ethanol is not affected by incremental feeding.

c) List of papers prepared and talks given

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W.M. Ingledew. "Yeasts for Production of Fuel Ethanol" In: The Yeasts Vol 5. Edited by: A.H. Rose and J.S. Harrison. Academic Press (in press)

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K.C. Thomas, and W.M. Ingledew. 1989. Novel Improvements in Production of Alcohol from Surplus Grain. Agricultural Development Fund Conference - Developing Innovations for Tomorrow, October, Regina.

Diefenbaker Center Display on Advanced Technology in Alcohol Production, Summer, 1989

E. O'Connor-Cox, Ph.D Thesis. Optimization of Oxygen and Nitrogen Concentrations in High Gravity and Very High Gravity Lager Fermentations. 1989

Finalist, 1989 High Technology Entrepreneur Award for Innovation or Scientific Breakthrough - Peat Marwick/Sask. Science and Technology/SATMA.

Winner, 1990 High Technology Entrepreneur Award for Innovation and Scientific Breakthrough - Peat Marwick Thorne/Sask. Science and Technology/SATMA.

Nominee, 1990 Bell Canada Forum Award (Corporate Higher Education Forum)

Nominee, 1990 Hoechst Canada/GATE Innovation Award

Post-Secondary Excellence Award - Nov, 1989 to University of Saskatchewan by Molson Breweries of Canada in recognition of work of W.M. Ingledew - made to establish an annual lectureship in Applied Microbiology and Food Science Department

Lecturer - Advanced Technology - Rotary Club's International Student Visitation to Province of Saskatchewan, May 8, 1990.

W.M. Ingledew. 1988. Yeast Biochemistry. 8th Annual National Short Course for Alcohol Distillery Operators, Managers and Chemists. Alltech Biotechnology Corporation. October, Lexington.

Cognate papers published during the period of this grant but funded elsewhere

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Papers presented at meetings;

B. Ziola, M. Whiting, S. Gares, and W.M. Ingledew. 1989. Enzyme Immunoassay (EIA) of Bacterial Contaminants in Breweries. Society of Industrial Microbiology, August, Portland.

J.K. Paik, N.H. Low, and W.M. Ingledew. 1990. Relationship of Chemical Composition to the Fermentability of Malt Extracts. American Society of Brewing Chemists, May, Fort Lauderdale.

J.K. Paik, W.M. Ingledew, and N.H. Low. 1990. Detection of Unlabelled Adjuncts or Adulterant Carbohydrates in Malt Extracts by High Performance Liquid Chromatography. Canadian Institute of Food Science and Technology, June, Saskatoon.

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E.S.C. O'Connor-Cox, and W.M. Ingledew. 1989. Fermentation Performance Related to the Timing of Oxygen Supply. American Society of Brewing Chemists, April, Denver.

d) Statement of expenditures

a statement from the University will be forwarded shortly

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